REMARKS

In response to the Office Action of August 24, 2004, Applicant is filing concurrently herewith a Request for Continued Examination, along with this Preliminary Amendment. In this Preliminary Amendment, claims 3-6 and 8-10 are amended for clarification purposes as well as to respond to the Examiner's previously rejections. In view of the following arguments as well as the above-identified amendments, reconsideration of this application is respectfully requested.

More particularly, in the Office Action, the Examiner objected to claims 8-10 due to a spacing error between the terms "the" and "adrenomedullin". Additionally, claims 8-12 were rejected under 35 U.S.C. § 112 as allegedly being indefinite. According to the Examiner, it was unclear in the subparagraphs of claims 8-10 how the subparts were incorporated into independent claim 7. In order to clarify this issue, claims 8-10 have been amended to depend from claim 1 or 2 as opposed to claim 7, 8 or 9, as previously set forth. Consequently, Applicant submits that claims 8-10, as now amended, overcome the deficiencies noted by the Examiner.

Additionally, in the Office Action, the Examiner rejected claims 1-10 under 35 U.S.C. § 102(b) as allegedly being anticipated by a new reference (i.e., Yallampalli, et al.), WO9734922. However, Applicant traverses this rejection.

In this regard, claim 1-10 are believed to be novel over the disclosure of Yallampalli, et al. for several reasons.

The Examiner asserts that the reference discloses that CGRP and adrenomedullin (hereinafter, AM) is effective for premature labor and that CGRP administration leads to an inhibition of uterine contraction and reduces uterine activity during pregnancy, thus the administration of CMRP and AM would inherently result in an inhibition of spontaneous myometrial contraction or bradykinin induced contraction. However, as described in Physiol Rev., 84, pp 903-934, 2004, a copy of which is attached as Exhibit A, the homology between AM and CGRP of active sites is about 25% (see p905, the last line of the right column), and their respective expression levels differ in different organs. Also it is well known in the art that their effects are different despite belonging to the same protein family. Therefore, even if Yallampalli, et al. describes the effect of CGRP, it could not be said that Yallampalli, et al. discloses the

effect of AM on spontaneous myometrial contraction or bradykinin induced contraction. Consequently, Applicant requests that this rejection be withdrawn.

Furthermore, in paragraph 6 of the Office Action, the Examiner has rejected claims 11-15 under 35 U.S.C. § 103(a) as being unpatentable over Yallampalli, et al., as applied to claims 1-10 above, and further in view of Kitamura, et al. (U.S. Patent No. 5,639,855). Applicant also traverses these rejections.

As mentioned above, the Examiner alleges that the administration of CMRP and AM would inherently result in an inhibition of spontaneous myometrial contraction or bradykinin induced contraction. However, Yallampalli, et al. indicates that, as shown in FIG. 7, CGRP suppresses the delivery rate associated with increased PGF2α and that CGRP inhibits natural myometrial contraction by PGR2α. On the other hand, AM of the present invention does not show inhibition of myometrial contraction by PGF2α as shown in Example 4, indicating that the mechanism of AM differs from that of CGRP. That is, AM could inhibit abnormal myometrial contraction via inflammatory mediator such as bradykinin, not myometrial contraction by PGF2α as seen in normal labor. CGRP does NOT have such an effect. In fact, Yallampalli, et al. indicates that CGRP only inhibits myometrial contraction in the later term of pregnancy (Day 18) and thus might inhibit normal labor. However, AM of the present invention does not inhibit myometrial contraction by PGF2α, oxytocin or the like and therefore does not inhibit normal labor.

From the above, it is demonstrated that the mechanisms of AM and CGRP are clearly different and that the diseases that AM is to be applied to are also different. It has been found, for the first time, that AM is effective for treating myometrial contraction due to bradykinin as mentioned in the above and that AM is useful for treating premature labor due to abnormal myometrial contraction.

Moreover, Kitamura, et al. does not disclose the method of the amended claims, that is inhibiting spontaneous myometrial contract or bradykinin-induced contraction, or premature labor, miscarriage, parturition prior to cesarean section or dysmenorrheal caused by spontaneous myometrial contraction or bradykinin-induced contraction, even if Kitamura, et al. describe the sequence of AM *per se*.

Therefore, in light of the description of the references to inhibition of uterine contraction in Yallampalli, et al. and Kitamura, et al., the claimed inventions are not obvious. Accordingly, Applicant requests that these rejections be withdrawn.

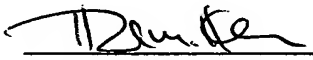
In view of the above amendments and arguments, it is respectfully submitted that the present application is now in condition for allowance. Hence, withdrawal of the rejections and issuance of a Notice of Allowance is requested.

In the event the Examiner considers personal contact advantageous to the disposition of this case, he/she is hereby authorized to contact Richard M. Klein at the telephone number listed below.

Respectfully submitted,

FAY, SHARPE, FAGAN,
MINNICH & McKEE, LLP

January 24, 2005
Date



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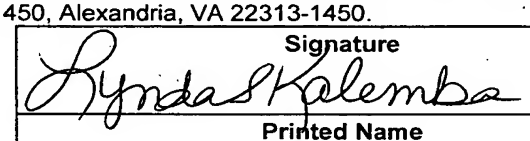
EV 494998907 US	
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EXHIBIT A

PTO/SB/08a (06-03)
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT(S)

(Use as many sheets as necessary)

Sheet 1 of 1

COMPLETE IF KNOWN	
Application Number	10/030,298
Filing Date	December 21, 2001
First Named Inventor	Yanagita
Art Unit	1654
Examiner Name	Gupta
Attorney Docket No.	YAMZ 2 00014

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.	Document No. Number-Kind Code (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document
	AA	US-		
	AB	US-		
	AC	US-		
	AD	US-		
	AE	US-		
	AF	US-		
	AG	US-		
	AH	US-		
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FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.	Foreign Patent Document Country Code-Number Kind Code (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	T
	AM				
	AN				
	AO				
	AP				

OTHER - NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume/issue number(s), publisher, city and/or country where published	T
	AQ	BRAIN, Susan D. and GRANT, Andrew D., *Vascular Actions of Calcitonin Gene-Related Peptide and Adrenomedullin*, Physiol. Rev., 84, pp. 903-934, 2004.	
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Examiner Signature		Date Considered	

Physiol Rev
84: 903–934, 2004; 10.1152/physrev.00037.2003.

Vascular Actions of Calcitonin Gene-Related Peptide and Adrenomedullin

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Brain, Susan D., and Andrew D. Grant. Vascular Actions of Calcitonin Gene-Related Peptide and Adrenomedullin. *Physiol Rev* 84: 903–934, 2004; 10.1152/physrev.00037.2003.—This review summarizes the receptor-mediated vascular activities of calcitonin gene-related peptide (CGRP) and the structurally related peptide adrenomedullin (AM). CGRP is a 37-amino acid neuropeptide, primarily released from sensory nerves, whilst AM is produced by stimulated vascular cells, and amylin is secreted from the pancreas. They share vasodilator activity, albeit to varying extents depending on species and tissue. In particular, CGRP has potent activity in the cerebral circulation, which is possibly relevant to the pathology of migraine, whilst vascular sources of AM contribute to dysfunction in cardiovascular disease. Both peptides exhibit potent activity in microvascular beds. All three peptides can act on a family of CGRP receptors that consist of calcitonin receptor-like receptor (CL) linked to one of three receptor activity-modifying proteins (RAMPs) that are essential for functional activity. The association of CL with RAMP1 produces a CGRP receptor, with RAMP2 an AM receptor and with RAMP3 a CGRP/AM receptor. Evidence for the selective activity of

the first nonpeptide CGRP antagonist BIBN4096BS for the CGRP receptor is presented. The cardiovascular activity of these peptides in a range of species and in human clinical conditions is detailed, and potential therapeutic applications based on use of antagonists and gene targeting of agonists are discussed.

I. HISTORY AND DISCOVERY

A. Calcitonin Gene-Related Peptide: A Novel Neuropeptide

Calcitonin gene-related peptide (CGRP) is a 37-amino acid neuropeptide that was identified in 1982 by molecular biological techniques. It was discovered when alternative processing of RNA transcripts from the calcitonin gene were shown to result in the production of distinct mRNAs encoding CGRP. The calcitonin mRNA predominates in the thyroid while the CGRP-specific mRNA appears to predominate in the nervous system (299). A human form of CGRP was isolated from thyroid tissue of patients with medullary thyroid carcinoma (259). CGRP is highly expressed in certain nerves (305) and is now known to belong to a family that includes the more recently discovered peptides adrenomedullin and amylin (see Fig. 1). This family belongs to a larger family of peptides that includes calcitonin. Calcitonin is a potent inhibitor of bone resorption, acting via receptor-mediated inhibition of osteoclast function (see Ref. 160). The overall effect of CGRP on bone resorption is unclear, although it can inhibit osteoclast activity (see Ref. 149), but it is best known for its potent cardiovascular effects.

CGRP is distributed throughout the central and peripheral nervous systems and exhibits a range of biological effects on tissues including those associated with gastrointestinal, respiratory, endocrine, and central nervous systems (101, 151, 235, 261, 293, 365, 382, 383). Its most widely described effects are associated with the cardiovascular system and are the subject of this review. CGRP is a potent arterial and venous vasodilator. In all cases, the relaxation to CGRP is blocked by the adminis-

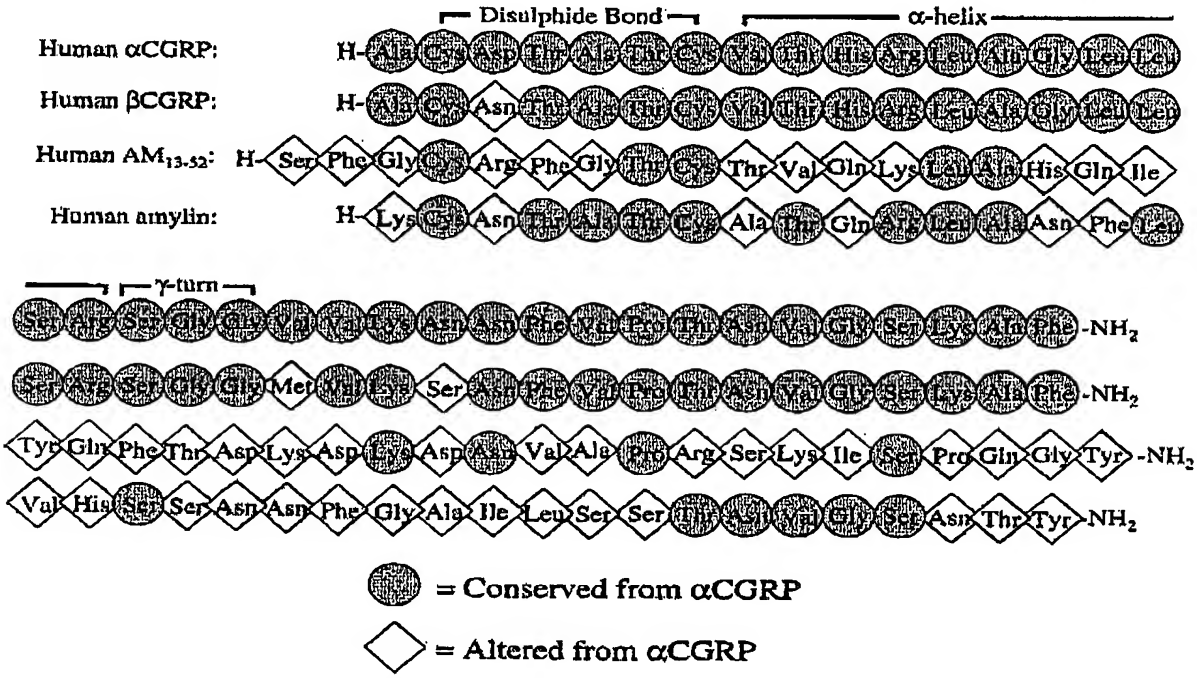

FIG. 1. Amino acid sequences of the human forms of α- and β-calcitonin gene-related peptide (CGRP) and related peptides (AM_{13-52} and amylin), showing the areas of conserved and altered residues compared with αCGRP. The conserved amino acid residues are shown as solid circles and the residues that are altered as open diamonds. The structure of AM_{13-52} is shown, as the biological activity is found within these residues (see sect. II for further explanation).

tration of the peptide fragment $CGRP_{8-37}$, a CGRP receptor antagonist, indicating a specific receptor-mediated mechanism. At a cellular level the effects of CGRP are mediated by stimulation of adenylate cyclase and accumulation of cAMP (see previous reviews in Refs. 20, 28, 34, 296). The microvasculature appears most sensitive to the physiological effects of CGRP. CGRP is one of the most potent microvascular vasodilator substances identified to date, with a potency ~10-fold greater than the prostaglandins and 100–1,000 times greater than other classic vasodilators (e.g., acetylcholine, adenosine, 5-hydroxytryptamine, and substance P). This effect was first demonstrated in skin, where femtomole-picomole amounts of injected CGRP were able to induce reddening due to local microvascular dilation. In addition to its great potency, CGRP also differs from other vasodilator substances in that it has a particularly long duration of action. A dose of 15 pmol injected into human skin produces an erythema that lasts for 5–6 h (31). Further studies of CGRP have shown that its vasodilator activity extends to a wide variety of tissues and organs from other species, with particularly potent activity in the cerebral circulation, suggesting that it plays a role in the vasodilatation associated with the pathology of migraine (121). This in turn has highlighted a need to identify small nonpeptide receptor antagonists.

B. Adrenomedullin: A Tissue-Derived Peptide

Adrenomedullin (AM) is a 52-amino acid peptide, which was discovered in 1993. It was isolated from human pheochromocytoma cells and was identified by its ability to stimulate cAMP production in platelets (197). It was soon realized that AM is produced by a wide range of cells including vascular endothelial and smooth muscle cells, especially upon stimulation with inflammatory cytokines (334–336). AM shares some of the cardiovascular activity of CGRP and thus may contribute to altered vascular function in disease. Only the terminal 40 amino acids of AM (AM_{13-52}) are required for its biological activity. AM, or the active fragment AM_{13-52}, has vasodilator and hypotensive effects but is 3–30 times less potent than CGRP (59, 132). Several reviews have discussed the clinical cardiovascular activities of AM in detail (94, 170), while the cellular and molecular biology of this peptide are reviewed by Lopez and Martinez (226). AM acts as a paracrine factor that can influence growth and development, renal effects, and endocrine (although it does not act as a hormone) activities. These properties have been extensively reviewed elsewhere (145).

A further peptide product encoded by the AM gene is pro-AM NH_2-terminal 20 peptide (PAMP; Ref. 198). The precursor peptide pro-AM can be cleaved to produce AM and PAMP. PAMP is ~30–100 times less potent than AM as a hypotensive agent in the rat (45, 325) but has been the subject of considerably less investigation. PAMP has been shown to be ~60 times less potent than AM in the human forearm and is suggested to be of less importance in the regulation of blood pressure and flow (381).

C. Amylin: A Pancreatic Peptide

Amylin, or islet amyloid polypeptide (IAPP), is a 37-amino acid peptide that shares some structural homology with CGRP and AM (as shown in Fig. 1), as well as similarities in some of its biological activities and will be mentioned briefly in this review. Amylin was discovered as amyloid deposits in the pancreas of non-insulin-dependent diabetics (48, 65, 377). It has some vasodilator activity (32, 111), but its major physiological effect is regulating glucose metabolism. It is secreted with insulin from pancreatic β-cells after meals. Amylin acts in the opposite manner to insulin with respect to glycogen synthesis and glucose uptake into muscle (64, 219; see Ref. 154 for review). Amylin has also been suggested to have roles in renal development and islet enlargement and may play a role in the development of the kidney (385). It is suggested that amylin decreases food intake in rats (47, 258) and that salmon calcitonin, which has a high affinity for amylin binding sites, can also mediate this effect (231). Furthermore, it has been proposed that amylin agonists could be beneficial as adjunct therapy in type 1 and certain cases of type 2 diabetes, where endogenous amylin is lacking (379).

II. STRUCTURE

The amino acid sequences of CGRP, AM_{13-52}, and amylin are shown in Figure 1. The tertiary structure of CGRP has not been conclusively determined. In 1991, Breeze et al. (33) produced data from NMR and distance geometry studies suggesting that CGRP consists of a characteristic NH_2-terminal disulfide bridge-linked loop between cysteines Cys^2 and Cys^7, followed by an alpha-helix in amino acids Val^8-Arg^{18} and a poorly defined turn between amino acids Ser^{19}-Gly^{21}. Later, also using NMR and molecular modeling techniques, Boulanger et al. (25) produced a suggested structure for CGRP with a disulfide-linked loop between residues Cys^2 and Cys^7, a helix segment between residues Val^8 and Leu^{16} (rather than Arg^{18}), and defined the turn between residues 19 and 21 as a γ-type. The COOH and NH_2 terminals of the peptide can interact independently with its receptors in that the $CGRP_{8-37}$ fragment is an antagonist whilst $CGRP_{1-7}$ is important for efficacy (see Ref. 20). There are two isoforms of CGRP for most species (α and β) that exhibit similar functional activities and differ by between one and three amino acids (7, 259, 305; see Fig. 1). AM_{13-52} has 25%

structural similarity with CGRP, while amylin shares 46–50% structural homology (304). AM_{13-52} has ~22% structural homology with amylin (see Fig. 1).

III. DISTRIBUTION AND REGULATION OF GENES AND PEPTIDES

A. CGRP: A Wide Distribution

CGRP is widely distributed in the central and peripheral nervous systems (28, 83, 316, 383). It is primarily located in small, unmyelinated sensory C fibers and myelinated Aδ fibers in the periphery, where it is most usually found in nerves that are closely associated with blood vessels. CGRP is often colocalized with other peptides in C fibers, especially the tachykinins substance P and neurokinin A (230). It is considered that of the two forms, αCGRP, encoded by the calcitonin gene, is the more abundant and found in discrete areas of the central and peripheral nervous system. In comparison, βCGRP, which differs from αCGRP by three amino acids in the human (see Fig. 1), is primarily located in the gut, in sites including those of enteric nerves (265) and the pituitary gland (288). βCGRP is formed from a separate gene that does not produce calcitonin (5, 7). Both forms of CGRP possess similarly potent biological activity in terms of vasodilator activity (29), although there are proposed differences in their receptor-mediated effects (see sect. IVC). In general, it is the effects of αCGRP that are discussed in this review.

CGRP has been identified at many sites complementary to its activity as a vasoactive mediator (see Ref. 150 for review). For example, CGRP-containing nerves innervate smaller arteries, where innervating nerve terminals can pass into the vascular smooth muscle layer. This allows CGRP to be released where it can have profound effects on arteriolar dilatation and on the microvasculature. CGRP-containing nerves also innervate venous tissues, but its activity on these tissues is less well documented. The distribution of CGRP-containing nerves has been studied in most tissues but has probably been most extensively reviewed with respect to pathophysiological function in the cerebral circulation (83). CGRP is released from sensory fibers originating in the trigeminal ganglia and acts to dilate cerebral vessels (119). The gut has also been intensively studied: here CGRP released from spinal afferents acts to dilate mucosal blood vessels and may protect against the acidic environment. It is possible that CGRP-containing vagal afferents, which originate from nodose ganglia, have a preferential nociceptive role (152).

CGRP has been suggested to be more abundant in common laboratory species than in humans (380). However, CGRP is localized in nerves in human coronary arteries and veins, especially at the adventitial-medial border, and has potent relaxant effects on arteries (127, 308). Low (picomolar) levels of immunoreactive CGRP have been detected in the plasma of healthy volunteers (117), with levels elevated during pregnancy (332). It is generally considered that the levels of CGRP detected in plasma are likely to be due to leakage after localized release rather than a specific systemic function. However, CGRP has the ability to decrease blood pressure and increase heart rate when given by intravenous administration to human volunteers (113), indicating that if sufficiently high plasma levels of CGRP are reached, systemic vasoactive effects can be triggered.

The regulation of CGRP production is poorly understood. Plasticity occurs at the level of the ganglia, for example, in models of peripheral axotomy, enteritis (190), and inflamed arthritic joints (77). In each case there is an associated increase in CGRP production in the ganglia. One factor of potential importance in influencing plasticity is nerve growth factor (NGF), which has an important role in the growth and maintenance of sensory nerve function (350). At a cellular level, NGF upregulates CGRP via a cAMP/ras responsive element (106), and via a constitutively active mitogen-activated protein kinase (MAPK) kinase (MEK; Ref. 82). In some experiments the upregulation of CGRP has been associated with nerve sprouting, an indication of NGF activity (56). Furthermore, upregulation of CGRP production in the dorsal root ganglia by NGF has been linked to restoration of the endogenous microvascular activity of CGRP in diabetic skin (72), and in promoting CGRP expression in the spontaneously hypertensive rat (341). It is possible that NGF acts as a rescue factor in times of vascular stress.

The release of CGRP from peripheral nerves was demonstrated at an early stage (73, 370). A classical mechanism leading to the release of sensory neuropeptides is that mediated by capsaicin, but the endogenous significance of this release mechanism remains unproven. CGRP immunoreactivity increases in the plasma after the administration of capsaicin, although the elevation is short lived (396). Capsaicin acts via vanilloid (TRPV1) receptors on sensory C and Aδ fibers to increase permeability to cations (42). This leads to nerve depolarization, release of neuropeptides, and, in time, their depletion from sensory nerves. Indeed, long-term treatment with capsaicin to deplete the sensory neurogenic component has been exploited in a beneficial manner to treat a range of vascular conditions in humans (see Ref. 342). Low pH and heat are also associated with the activation of the capsaicin receptor, leading to a release of CGRP (e.g., Refs. 115, 193). This may be relevant to the release and contribution of CGRP in models of cardiovascular ischemic inflammation, where localized acidity and increased heat are observed. It has been recently suggested that a range of endogenous agents may also act to stimulate this receptor. These include anandamide (405) and leukotri-

ene B_4 (158). Other substances considered to be involved in mediating the release of CGRP include kinins and prostaglandins (9, 124, 168) and NO (22, 176). It has been suggested that under certain circumstances, such as septic shock, mediators can act in a synergistic manner to potentiate CGRP release (374). The direct influence of inflammatory cytokines remains unclear, although it is suggested that interleukin (IL)-1β can act in a time- and protein synthesis-dependent manner to increase CGRP release from dorsal root ganglion neurons via a protein kinase C-dependent mechanism (156). Proteinase-activated receptor-2 is a member of a novel subfamily of G protein receptors that are activated by proteolysis and present on sensory nerves where they stimulate CGRP release (331). The functional and pathological importance of this response is not yet known (367).

Presynaptic/prejunctional receptors on the sensory nerves themselves also play an important role in modulating CGRP release. Evidence indicates a range of such receptors that include those for opioids, 5-hydroxytryptamine (5-HT_1 receptor), γ-aminobutyric acid ($GABA_B$ receptor), histamine (H_3 receptor), neuropeptide Y, somatostatin, vasoactive intestinal polypeptide, purines, and galanin (see Refs. 15, 27, 284 for reviews). A recent study suggests that excitatory CGRP receptors are also present on sensory neurons within the dorsal root ganglia, coupled to an increase in intracellular calcium, and these may act as stimulatory autoreceptors (315). There is evidence in the rat mesentery that reciprocal interactions can occur between the noradrenergic constrictor system and the sensory system. It has been shown that stimulation of α_2-adrenoceptors, located presynaptically on sensory neurons, acts to inhibit CGRP release (188), with CGRP also inhibiting the release of norepinephrine from sympathetic nerves (189). These results are indicative of an important role for CGRP in the regulation of peripheral blood flow.

In the circulation, CGRP has a half-life of ~7–10 min in humans (205, 333). There is not an obvious mechanism for CGRP metabolism, and it is probably broken down via a number of routes. Mast cell tryptase has a potent effect in cleaving CGRP into inactive fragments, both in vivo and in vitro. This mechanism has been clearly demonstrated in extravascular sites, e.g., skin (30). In addition, CGRP can compete with substance P for breakdown by an enzyme in the central nervous system (279). In contrast to substance P, CGRP seems to be a poor substrate for neutral endopeptidase, so this pathway is probably less important as a route for CGRP degradation in peripheral tissues (185). A matrix metalloproteinase II has the ability to metabolize CGRP and remove its vasodilator activity (99). Finally, there has also been a suggestion that CGRP may be taken back up into sensory nerve terminals after repolarization, at least in vitro (313).

B. AM: Upregulation in Disease

The AM gene is located on chromosome 11 in humans (163). Unlike CGRP, AM is primarily produced by nonnervous tissue, especially endothelial (334) and vascular smooth muscle cells (336). AM mRNA is thus primarily found in most areas where abundant microvascular vessels exist (e.g., heart, lung, kidney, and cerebral vasculature; Refs. 159, 209). In particular, AM is produced in the atrium of the heart (170). The normal circulating levels of AM are in the low picomolar range, but levels are increased in disease and certain physiological conditions, including pregnancy (138). Studies have revealed increased circulating levels of AM in cardiovascular diseases such as hypertension and stroke, as well as septic shock, and it has been suggested that the increased levels are in proportion with disease severity (302, 375).

AM is constitutively released from endothelial cells, and its release is regulated entirely at the level of protein expression (334). The increase in AM plasma levels in disease is associated with an increase in AM gene expression in tissues, especially vascular and smooth muscle cells (161, 334–336), as discussed above. Increased AM production probably contributes to the vascular component of inflammatory disease, particularly as the major cytokines tumor necrosis factor (TNF)-α and -β and IL-1α and -1β are potent stimulators of upregulation of the AM gene in endothelial and smooth muscle cells (334–336). Interestingly, AM has been shown to potentiate IL-1-stimulated inducible nitric oxide synthase (NOS) and thus nitric oxide (NO) synthesis, although AM does not appear to have a direct effect on inducible NOS generation (139). Other factors shown to increase the mRNA and/or synthesis of AM in vascular smooth muscle cells include the adrenocortical steroids and thyroid hormones (252). In addition, the finding that shear stress promotes AM mRNA production in human endothelial cells is of relevance to vascular dysfunction (62). The regulation of the production and secretion of AM in the cardiovascular system has been recently reviewed (93). An adrenomedullin binding protein, originally known as complement factor H, has been discovered (89). This protein has been suggested to facilitate the presence of high concentrations of AM at receptor sites in tissues, and possibly also to modulate the degradation of AM (291).

The increase in tissue levels in disease has been most extensively studied in septic shock (147, 274). During infection, bacterial and viral products such as bacterial lipopolysaccharide (LPS) stimulate release of cytokines such as TNF-α and IL-1β. These cytokines in turn stimulate the host defense against microbial pathogens, and excessive production of these cytokines may be responsible in part for the fatalities observed during sepsis and systemic inflammatory response syndrome (SIRS; Ref.

17). Thus a link has been established between LPS, cytokines, and the increased levels of AM in sepsis and SIRS patients (see sect. IXE).

C. Amylin: Relevance to Diabetes

Amylin was primarily found as a major peptide constituent of amyloid deposits in the islet β-cells in the pancreas of type 2 diabetics (65), and in the amyloid deposits associated with pancreatic tumors (378). Substantially lower amounts of amylin are found in nerves, but interestingly, it is constitutively expressed in CGRP-containing neurons. It is upregulated in a transient manner in a model of joint inflammation in the rat paw (264). This may be related to the early inflammatory component in this model. Amylin has also been localized to the pyloric antrum of the stomach, duodenum, jejunum, ileum, and colon in the rat (10).

Amylin is cosecreted with insulin in response to glucose, but the relative amount of each peptide may vary, depending on situation. Like insulin, it is lacking in type 1 diabetes, in keeping with the loss of the pancreatic cells. In obese subjects, amylin mirrors release of insulin as insulin resistance develops, but is deficient in type 2 diabetes (228), as insoluble amyloid fibrils are thought to develop. Evidence has recently been provided that a mutation in the enhancer region of the amylin promoter may be related to the development of type 2 diabetes (277).

IV. RECEPTORS

The knowledge, as discussed above, of shared and individual activities of these peptides has helped, together with complex results from both functional and molecular experiments, to fuel considerable debate and interest about the receptors for this family of peptides. The understanding of these receptors is still at an early stage, and the information available to date is described below. CGRP acts on its own CGRP receptor whilst AM can act via both CGRP and AM receptors to mediate its vasorelaxant effects, as will be discussed more fully in sections V–VII.

The existence of two receptors, CGRP1 and CGRP2, was originally proposed in the late 1980s, with the CGRP1 receptor being the predominant mediator of cardiovascular effects. This receptor classification was developed as a consequence of pharmacological studies carried out with different agonists and antagonists in a range of tissue preparations, especially the positive inotropic effect in the guinea pig or rat atrium for determination of CGRP1 receptor activity, and the inhibition of electrically evoked twitch responses in the rat vas deferens for determination of CGRP2 receptor activity (69, 70, 81). The 30-amino acid fragment of CGRP, $CGRP_{8-37}$, is an antagonist showing preference for the CGRP1 receptor (54). In contrast, linearized CGRP analogs such as diacetoamidomethyl cysteine CGRP {[Cys(ACM)2,7]hαCGRP} are considered to show preferential agonist potency for the CGRP2 receptor. [Cys(ACM)2,7]hαCGRP is formed by reduction of the disulfide bond of CGRP. In general, receptors that can be antagonized by $CGRP_{8-37}$ with an approximate pK_b value of 7.0 are designated as CGRP1 receptors, while those that $CGRP_{8-37}$ block with a pK_b of 6 or less are classified as CGRP2 receptors (293, 299). However, more recent studies have questioned the selectivity of [Cys(ACM)2,7]hαCGRP for the CGRP2 receptor and suggested that it also exhibits potent activity at the CGRP1 receptor (79). This classification of receptors still holds true today in that experiments with peptidase inhibitors have not revealed reasons for the differences (173). However, the classification is not universally accepted, as recently debated by Poyner and Marshall (294). They describe how mean pA_2 values that range between 8.1 and <5 in 11 different rat tissues do not allow themselves to be readily divided into two receptor groups (294).

Evidence for an AM receptor was initially obtained from functional studies where the responses were not inhibited by $CGRP_{8-37}$ (e.g., in the rat; Ref. 270). Eguchi et al. (88) suggested that human AM_{22-52} is an antagonist of the AM receptor. There is some confusion in the literature, in that some studies (e.g., in the perfused hindlimb vascular bed of the cat) show that AM_{22-52} did not antagonize vasodilator responses to AM, but did inhibit CGRP responses (46). However, in later studies, AM_{22-52} was found to selectively antagonize rat cerebral vasodilatation (76) and has slowly become established as a weak antagonist of AM responses (see Ref. 112), thus strengthening the concept of a specific AM receptor.

A. Cloning of CGRP Receptors

Several members of the CGRP family of receptors have been cloned in recent years. In 1995 an orphan receptor commonly known as L1 (see Ref. 13) was suggested to be an AM receptor (183), although little supporting evidence has been provided to date (192). A second receptor, a putative CGRP receptor, RDC, that had originally been shown to increase cAMP in response to CGRP (184) has also not been supported in the literature (173, 248).

Rat calcitonin receptor-like receptor (CL) was cloned in 1993 (275). Human CL was cloned 2 years later and consists of 461 amino acids with 7 transmembrane domains; 91 and 56% of the amino acids were found to be identical to the rat orphan calcitonin receptor-like sequence and the human calcitonin receptor, respectively (103). However, the receptor did not bind CGRP in the cells studied and was considered an orphan receptor. A

major breakthrough was made in 1996 when Aiyar et al. (4) cloned and characterized cDNA encoding the hCGRP1 receptor. Interestingly, the cloned receptor demonstrated significant peptide sequence homology with CL. Furthermore, the cDNA was expressed in a stable manner in human embryonic kidney 293 cells (HEK293) with associated specific, high-affinity binding sites for CGRP that displayed functional properties very similar to the human CGRP1 receptor. CGRP induced a 60-fold elevation in cAMP production that was inhibited in a competitive manner by $CGRP_{8-37}$ (4). These results were this time confirmed by two other groups using rat (134) and porcine CL (90). However, in the same study, Han et al. (134) found that CL expressed in COS-7 cells failed to produce a functional receptor, so they concluded that HEK293 cells must also possess an extra intrinsic factor necessary for the production of a functional receptor. It was not until the work of McLatchie et al. (248) was published that it was realized that a receptor activity-modifying protein (RAMP; a 148-amino acid peptide with a single transmembrane domain) was required to associate with CL to confer receptor activity (248).

B. CL and RAMPs

The CGRP/AM receptors that have been cloned and characterized to date consist of a seven-transmembrane G protein-coupled CL in association with one of three single membrane-spanning RAMPs (see Fig. 2). There is strong evidence from studies in cultured cells that CL, in combination with an appropriate RAMP, acts as a receptor for CGRP and adrenomedullin (e.g., Ref. 55). CL is a member of the B family of seven transmembrane G protein-coupled receptors. Members include, in addition to the calcitonin receptor, receptors for vasoactive intestinal polypeptide, pituitary adenylate cyclase activating polypeptide, and parathyroid hormone (318).

The RAMPs were described as a novel family of single transmembrane domain proteins (248). Three RAMPs have been identified (RAMP1, RAMP2, and RAMP3). The association of CL with RAMP1 produces a CGRP receptor (CGRP1) that is antagonized by the CGRP antagonist $CGRP_{8-37}$; CL with RAMP2 an AM (AM_1) receptor that can be antagonized by the weak AM peptide antagonist AM_{22-52} and CL with RAMP3 another AM receptor (AM_2). However, binding of CGRP to mouse CL with RAMP3 in COS-7 cells has also been reported (157). This finding was extended by Hay et al. (140), who demonstrated that while αCGRP is 15 times less potent than AM at activating the rat CL/RAMP3 receptor in COS-7 cells, βCGRP is only 2.5 times less potent. It is not clear whether βCGRP signaling via the AM_2 receptor plays an important role in vivo. The nomenclature for these receptor types has been recently discussed and standardized (295). The less widely distributed calcitonin receptor acts alone as a calcitonin (hCTR2) receptor but can also interact with RAMPs to produce a high-affinity receptor for amylin/calcitonin (58, 262) and CGRP (220). It is now suggested that RAMPs can also interact more widely with G protein-linked receptors that include the vasoactive intestinal polypeptide VPAC-1 receptor (57).

RAMP1, -2, and -3 have been identified in humans, rats, and mice. The same RAMPs in different species show >60% homology, but <30% homology exists between different RAMPs in the same species (330). The RAMPs interact with CL to provide an active receptor in the cell membrane and are essential in determining receptor specificity (23, 317). The extracellular NH_2 terminus of the RAMP is important for ligand binding. The deletion of residues 93–99 from RAMP2 and 58–64 from RAMP3 have

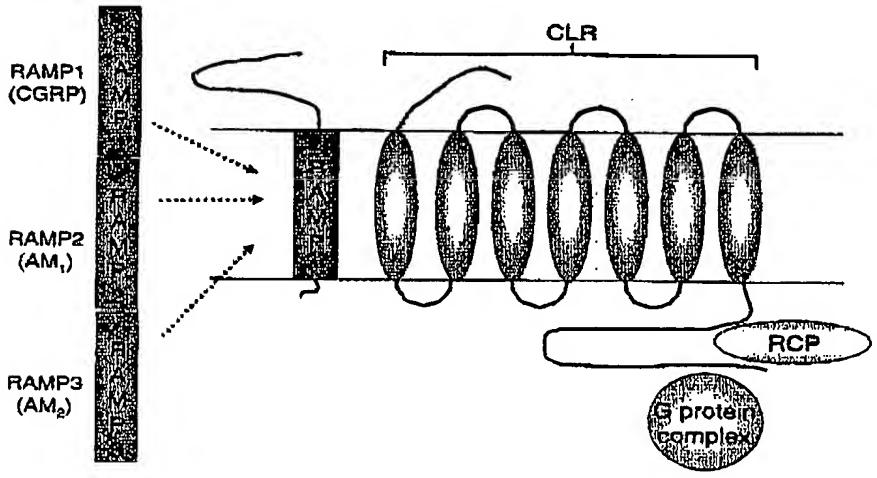

FIG. 2. The CGRP/adrenomedullin (AM) receptor model. The calcitonin receptor-like receptor (CL) component is common to all three receptors and is a G protein-coupled 7-transmembrane receptor. The three RAMP components are single transmembrane domain proteins. The active receptor is a functional heterodimer of one CL complexed with a RAMP, at the cell membrane. The interaction of RAMP1 with CL produces a CGRP receptor, RAMP2 with CL an AM receptor, and RAMP3 with CL a CGRP/AM receptor. The proposed receptor component protein (RCP), which is suggested to allow coupling to intracellular signaling pathways, is also included (see sect. IV for further explanation).

been each shown to substantially inhibit AM binding (211). In comparison, the short COOH terminus is not essential to activity, although loss of the W and Q amino acid residues adjacent to the membrane abolishes signaling activity (330). CL is widely distributed (130), so the expression pattern of the RAMPs is likely to determine the expression of functional CGRP and AM receptors. However, the regulation and functional relevance of the CL/RAMP interactions in vivo are still poorly understood. Studies from transfected endothelial cell lines indicate that the dynamic interactions between RAMPs can lead to competition between different RAMP types (263). It has been proposed that RAMP1 is dominant, leading to the preferential expression of a functional CGRP receptor when both RAMP1 and RAMP2 are present in a cell (36). In some cell types, such as cultured macrovascular (61) and dermal microvascular endothelial cells (181), RAMP2 is expressed to a much greater degree than RAMP1, producing functional AM receptors. Indeed, RAMP1 was not detected in human cerebral vascular tissue endothelial cells (281). Thus a substantial amount is known about the CGRP and AM receptors from elegant molecular and cell-based studies.

Studies of CGRP/AM receptor expression in various pathological conditions have revealed alterations in receptor components such as sepsis, indicating that receptor plasticity may play a role in pathophysiological states. Ono et al. (282) provide preliminary evidence in representative results from a model of sepsis, that the normally high expression of CL and RAMP2 in the mouse lung is substantially decreased at 12 h after induction of sepsis by LPS, but this has not been confirmed by Orman et al. (284) in rat sepsis. In addition, RAMP3 levels have been shown to be elevated in the late stages of sepsis (282, 284) and in a rodent model of chronic heart failure (67). In the latter model RAMP1 upregulation was also observed.

It is now accepted that while the CL is important for ligand binding, the RAMP proteins have roles in determining receptor phenotype and species selectivity. The trafficking activity of RAMP1 has been studied, and it is now realized that RAMP1, when expressed alone, is located in the endoplasmic reticulum and the Golgi mainly as a disulfide-linked homodimer (144). However, when found at the cell surface it is present as a heterodimer with CL. Recent evidence suggests that the RAMP protein is stablized at the cell surface when complexed with CL (57; see Fig. 2). The association of RAMP with CL leads to a poorly characterized noncovalent interaction. The coexpression of RAMP1 with CL as heterodimers at the cell surface in HEK-293 cells and studies of deletion mutants have revealed that residues 91–103 are important for high-affinity CGRP binding, although no individual residue was critical, while the deletion of residues 78–80 or 88–90 reduced AM binding (210). These authors suggest that the RAMPs probably influence binding by influencing the formation of a "receptor pocket" or by allosteric modulation of the conformation of the receptor. In elegant studies with hybrid CGRP receptors, Kane and co-workers (240) have described how chimeric RAMP constructs reveal that RAMP1 determines the species selectivity for receptor antagonists, and that a specific amino acid residue (tryptophan at position 74) is responsible. The binding of CGRP ligand and activation of the receptor is then associated with phosphorylation of CL and receptor internalization. The internalization process is typical of that associated with seven transmembrane receptors involving β-arrestin and clathrin-coated pit-mediated endocytosis (143). Interestingly, there is a lack of CL in some tissues, such as the cerebellum of certain species, where there is substantial CGRP binding and thus evidence for further receptors (52). In reality, the functional information has to be viewed alongside the molecular data, and this is not easy at the present stage.

The CGRP-receptor component (RCP; see Fig. 2) is a 17-kDa intracellular membrane protein that was cloned and shown to provide CGRP receptor activity to *Xenopus* oocytes (229). Antisense studies in NIH3T3 cells and immunocoprecipitation studies have implicated a role for RCP in driving the receptor-mediating response, by involvement in receptor coupling and stimulation of adenylate cyclase (96). However, this concept awaits further experimental confirmation, for example, through development of RCP knockout mice.

C. CGRP Receptor Antagonists

The CGRP antagonist $CGRP_{8-37}$ has been used since 1989 as a pharmacological tool to block CGRP1 responses (54). The peptide nature of this antagonist has limited its use, and there has been a requirement among researchers for a more stable nonpeptide antagonist. This requirement has accompanied the need to develop nonpeptide antagonists for possible treatment of pathological conditions, particularly migraine. Progress has been slow in that until 2000 the novel antagonists that had been developed had been considered either too difficult to use or, due to solubility problems, of insufficient potency for use. However, Boehringer patented a group of compounds as CGRP antagonists in 1998 and published results on the activity of one of these compounds BIBN4096BS in February 2000. BIBN4096BS, 1-piperidinecarboxamide, *N*-[2-[[5-amino-1-[[4-(4-pyridinyl)-1-piperazinyl]carbonyl]pentyl]amino]-1-[(3,5-dibromo-4-hydroxyphenyl)-methyl]-2-oxoethyl]-4-(1,4-dihydro-2-oxo-3(2H)-quinazolinyl)-,[*R*-(*R**,*S**)], is a competitive nonpeptide antagonist with potent antagonistic activity at the human CGRP1 receptor (79). It has an affinity (K_i) of 14.4 ± 6.3 pM for human CGRP1 receptors in SK-N-MC cells (a human neuroblastoma cell line) and 3.4 ± 0.5 nM for CGRP1 recep-

tors in spleen. This compares to 1.3 nM on SK-N-MC cells for $CGRP_{8-37}$ (87), so it is apparent that BIBN4096BS is much more potent. BIBN4096BS also displays potent selective antagonism of CGRP receptors in human and marmoset tissues with species selectivity (200-fold greater affinity compared with its binding in rodent tissues) when compared with common laboratory species. This was found to be due to a single amino acid difference between primate and rodent receptors, as recently described (240). Furthermore, there is evidence that this compound antagonizes human αCGRP more readily than βCGRP-induced positive inotropic effects, whereas $CGRP_{8-37}$ is a similar antagonist of both (386). BIBN4096BS has been used to learn more about the classification of receptors into the CGRP1 and -2 subclasses. Interestingly, like $CGRP_{8-37}$, BIBN4096BS shows an ~10-fold higher affinity for CGRP1 (blockade of positive inotropy in the rat left atrium) than CGRP2 receptors (inhibition of CGRP-evoked twitch in the rat vas deferens; Ref. 386). Furthermore, the authors also suggested that a novel receptor may exist in the rat vas deferens that is not blocked by $CGRP_{8-37}$ but at which AM and the linearized CGRP analog [Cys(Et)2,7]hαCGRP have potent activity. BIBN4096BS was found to block this receptor (386). More recently, studies using all-rat and rat-human combination AM_1 and AM_2 receptors in several different cell lines found that BIBN4096BS was unable to antagonize AM responses at doses up to 10 μM (140).

Experiments with BIBN4096BS have also aided in identifying and clarifying additional biological roles for CGRP. BIBN4096BS has been suggested as a potential therapy for sufferers of migraine, a condition linked to elevated secretion of CGRP. It potently antagonized CGRP-induced dilation of human temporal arteries (368), bovine middle artery and human meningeal, cerebral, and pial arteries (255). Dilation of these vessels is believed to be at least partially responsible for the pain suffered during a migraine attack (255).

A second compound from Boehringer patent WO98/11128, (4-(2-oxo-2,3-dihydro-benzoimidazol-1-yl)-piperidine-1-carboxylic acid [1–3,5-dibromo-4-hydroxy-benzyl)-2-oxo-2-(4-phenyl-piperazin-1-yl)-ethyl]-amide), Compound 1, has also been synthesized and studied. It is a weak antagonist of CGRP receptors. Binding data with SK-N-MC cells revealed pK_i values of 7.8, compared with 8.9 for $CGRP_{8-37}$ in displacing CGRP. It also weakly antagonized CGRP responses in human cerebral and guinea pig basilar arteries (87). However, this compound failed to inhibit the vascular relaxation induced by αCGRP, AM, and amylin in porcine coronary arteries (137), but does so in human coronary artery (136), whereas $CGRP_{8-37}$ is an effective antagonist in both tissues. Once again, these results underline the species and tissue selectivity that is now becoming associated with CGRP receptor antagonists.

An alternative nonpeptide CGRP receptor antagonist has been developed by GlaxoSmithKline (2). SB-273779, [*N*-methyl-*N*-(2-methylphenyl)-3-nitro-4-(2-thiazolylsulfinyl)-nitrobenzanilide], is selective for the CGRP receptor but is less potent than BIBN4096BS, with a K_i value of 310 ± 40 nM on SK-N-MC cells. In addition this compound was weak in competition studies with CGRP in rat and porcine lungs. The authors describe it as the first "cross-species" (i.e., comparable affinities in human, porcine, and rat tissues) nonpeptide CGRP antagonist (2).

V. VASODILATOR MECHANISMS

A. CGRP: Multiple Mechanisms

There are several mechanisms by which CGRP produces vascular relaxation, as discussed in earlier reviews (20, 28, 242). It is accepted that vasodilatation is mediated via the $CGRP_1$ receptor and blocked in a competitive manner by $CGRP_{8-37}$. Current evidence points to the existence of an NO- and endothelium-independent pathway, where CGRP administration correlates closely with a rise in intracellular cAMP ($[cAMP]_i$) (see Fig. 3). This mechanism is observed in the majority of tissues that have been studied to date (e.g., rat perfused mesentery, Ref. 133; cat cerebral artery, Ref. 86; porcine coronary artery, Ref. 393). The ability of CGRP to relax these tissues in the absence of an endothelium implies that it acts directly on the smooth muscle cells to stimulate adenylate cyclase, and this has been demonstrated in cultured smooth muscle cells (66, 148). The resulting rise in $[cAMP]_i$ activates protein kinase A (PKA), which probably phosphorylates and opens K^+ channels, leading to relaxation. Nelson et al. (271) first suggested an involvement of ATP-sensitive potassium channels in the vasodilator mechanism of CGRP in 1990. They showed that glibenclamide (an ATP-sensitive K^+ channel blocker) acted selectively to block the CGRP-induced response and that CGRP hyperpolarizes arterial smooth muscle. CGRP indirectly activates ATP-sensitive potassium currents via adenylate cyclase activation and cAMP generation in smooth muscle from porcine coronary artery (376) and guinea pig ureter (237). In other tissues, a role for K^+ channel activation in the relaxation to CGRP is evident, although coupling through activation of adenylate cyclase and cAMP generation has not been confirmed. In rat pial arterioles, vasodilatation to CGRP was inhibited in the presence of glibenclamide or charybdotoxin (a large-conductance Ca^{2+}-activated K^+ channel blocker; Ref. 153). In the mouse aorta (292) and rat renal microvasculature (301), vasodilatation to CGRP was partially inhibited by glibenclamide. Also, in the renal microvasculature, the vasorelaxant activity of CGRP could be mimicked by the application of pinacidil (an opener of ATP-sensitive K^+ channels) (301). Administration of bolus doses of CGRP to rats produced significant hypotension, which was potentiated by cromakalim (an

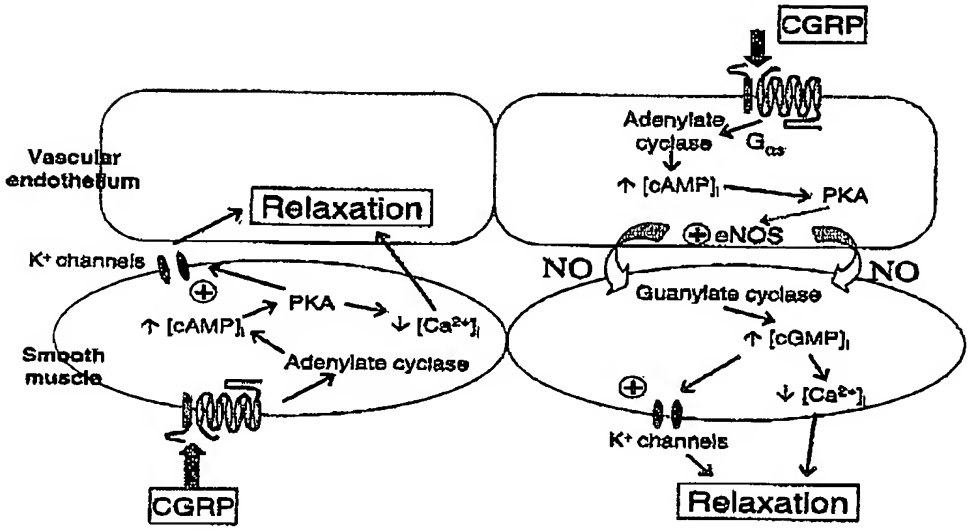

FIG. 3. The cellular mechanisms of vasodilatation to CGRP. *Left*: endothelium-independent vasodilatation to CGRP. Activation of CGRP receptors on smooth muscle cells is coupled to production of cAMP by adenylate cyclase. The increase in intracellular cAMP concentration ($[cAMP]_i$) then stimulates protein kinase A (PKA), which opens K^+ channels and activates Ca^{2+} sequestration mechanisms to cause smooth muscle relaxation. *Right*: endothelium-dependent vasodilatation to CGRP. CGRP interacts with receptors on endothelial cells and stimulates production of nitric oxide (NO). This is mediated via cAMP accumulation, although a direct effect of PKA on endothelial NO synthase (eNOS) is yet to be fully characterized. Diffusion of NO into adjacent smooth muscle cells, activating guanylate cyclase, then leads to relaxation.

opener of ATP-sensitive K^+ channels) and attenuated by glibenclamide (310). These data all indicate a role for K^+ channel activation in the relaxation to CGRP by vascular smooth muscle.

Endothelium-independent relaxation to CGRP occurs in the majority of tissues examined to date. Exceptions include the rat aorta, where the relaxation to CGRP occurs only in the presence of an intact endothelium and is attenuated by inhibitors of NO synthase, implying an NO-dependent mechanism (31, 125, 126). A similar endothelium-dependent mechanism of relaxation is also seen in human internal mammary artery (300) and rat pulmonary artery (384). A significant increase in both cAMP and cGMP occurs and is also dependent on the presence of endothelium (125). This implicates the release of NO from the endothelium, which then relaxes the smooth muscle cells through activation of guanylate cyclase and accumulation of cGMP (see Fig. 3). The importance of the increase in cAMP in the vascular endothelial cells remains to be determined, but it has recently been demonstrated that cAMP is able to stimulate endothelial NOS (eNOS) activity, leading to increased synthesis and release of NO (100, 298). The activation of eNOS via cAMP is probably mediated via PKA, as a recent study demonstrated that the catalytic subunit of PKA can phosphorylate and activate eNOS (39). It is a possibility that CGRP causes an increase in cAMP in the endothelial cells which leads to the NO release, and thus relaxation of the smooth muscle.

Both the signaling pathways described above are mediated through stimulation of adenylate cyclase to produce cAMP. This implies that the CGRP receptor is coupling to $G_s\alpha$ proteins. The CGRP receptor may also be able to stimulate intracellular activity through a different G protein. Aiyar et al. (8) reported that CGRP was able to activate phospholipase C (PLC) in HEK293 cells, leading to an increase in intracellular calcium via inositol trisphosphate (IP_3) activity. This increase in calcium occurred concurrently with the stimulation of adenylyl cyclase and accumulation of cAMP. Activation of PLC is considered to occur through $G_{q/11}\alpha$, rather than through $G_s\alpha$, suggesting that the activated CGRP receptor is able to interact with both types of G protein. If this mechanism is present in endothelial cells, it provides an alternative explanation for CGRP activation of eNOS (which is traditionally considered to be dependent on Ca^{2+}/calmodulin for activation) independently of cAMP accumulation. The possibility that CGRP receptors may be coupled to phosphatidylinositol turnover is supported by another study that found this secondary messenger pathway in skeletal muscle (216).

B. AM: Active on CGRP and AM Receptors

The mechanisms via which AM can elicit vascular relaxation are heterogeneous with respect to both species and vascular bed. They are incompletely understood, but known to involve both the CGRP and AM receptors as AM has been shown to induce vascular relaxation via either $CGRP_{8-37}$-sensitive or AM_{22-52}-sensitive mechanisms. Furthermore, in some tissues, there is evidence that AM can act via both endothelium-dependent (NO-dependent) and K^+ channel-dependent mechanisms (214, 307, 349, 393). In addition, there is some controversy in that different results have been obtained using the same tissue in different laboratories. One problem may be that the AM

antagonist AM_{22-52} (88), while being the best available, has been criticized for being weak and lacking in specificity (145). Thus the following acts as an overview, based on assessment of published information.

AM, like CGRP, has been shown to increase both cAMP (161, 186) and intracellular calcium levels in endothelial cells (324, 393). A similar effect of AM on cAMP levels has been reported in vascular smooth muscle cells, with a decrease in intracellular calcium levels, although results may depend on experimental conditions and tissue (14, 88, 393). There is evidence that AM acts to relax tissues via a CGRP receptor-dependent mechanism in the porcine coronary artery preconstricted with U46619 (393), in a range of arterial vessels from the dog, where the effect was largely unaffected by endothelial removal (268), in the rat coronary artery (324), rat cutaneous microvasculature (132), the rat isolated perfused kidney (142), and the rat mesenteric bed (278) to name a few. In addition, $CGRP_{8-37}$-resistant responses have been found in other tissues, where the weak AM antagonist AM_{22-52} blocked responses (e.g., rat cerebral microvessels, Ref. 202; rat smooth muscle cells, Ref. 88; and in the human coronary artery, Ref. 349).

A further mechanism that may be involved in AM signaling is suggested by Nishimatsu et al. (273). They examined the NO-dependent relaxation to AM in the rat aorta and identified the involvement of a phosphatidylinositol 3-kinase (PI3K)/Akt-dependent pathway in the stimulation of eNOS. The results showed that AM stimulated Akt activation in aortic endothelium via the Ca^{2+}/calmodulin-dependent route, an established stimulant of eNOS (366). This protein kinase was also implicated in the increased production of NO stimulated by shear stress, demonstrated by Dimmeler et al. (75). They found that serine phosphorylation of eNOS increased its Ca^{2+}/calmodulin sensitivity, and thus stimulated production of NO. Nishimatsu et al. (273) did not investigate the receptor involved in mediating NO-dependent relaxation in the rat aorta. However, in the human coronary artery, AM-induced, NO-dependent relaxation has been shown to be selectively blocked by AM_{22-52}, but not $CGRP_{8-37}$, indicating a role for a specific AM receptor. In this tissue it was suggested that AM stimulates vasodilatation via both NO and K^+ channels, with only a minor contribution of cAMP (349). The relevance of adenylate cyclase to NO production in response to AM stimulation is unclear, in that there is evidence for either a requirement for cAMP accumulation during relaxation in canine coronary blood vessels (400) or no requirement in bovine aortic endothelial cells (324).

There are thus a variety of mechanisms via which AM may induce vascular relaxation, and further research is required to fully understand them. However, it is clear that there is an overall similarity in the mechanisms of CGRP- and AM-induced relaxation. This is presumably related to the fact that both AM and CGRP are known to act via the same G protein-linked receptor (i.e., CL). The majority of the differences in intracellular signaling pathways activated by CGRP and AM are probably related to the different RAMPs that form their receptors. For example, RAMP2 and RAMP3 may be involved in modulating the migration of endothelial and smooth muscle cells, via cAMP-independent mechanisms. AM has been recently shown to inhibit the migratory activity of vascular smooth muscle cells, in a model where CGRP was inactive (107).

C. Amylin: Active Via the CGRP1 Receptor

The ability of amylin to act as a vascular relaxant of both macro- and microvessels is well established, although it is in the region of 100-fold less active than CGRP in vitro (16, 32, 131, 167) and is not considered to be involved in the regulation of blood pressure. The vasodilator effects of amylin appear to be mediated by the CGRP1 receptor as they are blocked by $CGRP_{8-37}$. The vascular relaxant effects of αCGRP, AM, and amylin have been compared in porcine coronary arteries. The responses were all blocked by $CGRP_{8-37}$, supporting the above findings. In this study a distinct amylin receptor (consisting of calcitonin interacting with a RAMP; see sect. *IV.B*) was sought, without success (137).

VI. CARDIOVASCULAR REGULATION

A. CGRP: Potent Vasodilator In Vivo

The vasodilator activity of CGRP has been studied extensively in the vasculature in vitro, and the section above highlights the importance of these studies for the elucidation of vasodilator mechanisms. Studies in vivo are also essential to determine the role of CGRP in cardiovascular regulation. The contribution of the various vasodilator mechanisms of CGRP to the vasodilatation in humans has been examined in a study in the human forearm where the ability of intra-arterial infusion of CGRP to stimulate a decrease in forearm vascular resistance due to vasodilatation is well established. The CGRP-induced vasodilatation induced by this intravascular source of CGRP was shown to be dependent, at least in part, on NO and not activation of K^+ channels (68). The role of cAMP was inconclusive, primarily as adenylate cyclase inhibitors are too toxic to administer in vivo. The intravenous administration of CGRP is associated with hypotension and positive inotropic and chronotropic responses in the rat (8, 19, 111). In comparison, intracerebroventricular injection of CGRP, as well as other members of the calcitonin family of peptides causes an increase in blood pressure in rats, due to sympathetic nerve stimulation and release of

the vasoconstrictor norepinephrine (102). However, the available evidence, as summarized below, suggests that CGRP does not have a role in the regulation of blood flow in the normal rat or mouse.

Studies with intravenously injected $CGRP_{8-37}$ in rodents have shown a lack of effect on basal blood pressure (111). A recent investigation in the anesthetized rat and conscious dog supports these results and shows that $CGRP_{8-37}$, at doses that antagonized the effects of CGRP, had no effect on either systemic blood pressure or regional vascular beds (323). This supports the hypothesis that endogenous CGRP acts in a local rather than a systemic manner to modulate blood flow. In addition to studies using CGRP receptor antagonists, elucidation of the role of CGRP in cardiovascular regulation has been aided by the creation of αCGRP knockout mice. However, the results from these mice have already caused some controversy. The first strain of αCGRP knockout to be created did not show any change in basal blood pressure (227). In contrast, αCGRP knockout mice produced by a different group showed pronounced increases in both systolic and mean arterial pressure, as measured by the tail cuff technique, or after implantation of a carotid artery cannula in conscious mice (108). In the two cases cited above, the knockout mice exhibited no obvious phenotypic differences from their wild-type counterparts, but the nature of the mode of deletion of CGRP differed. The mice studied by Lu et al. (227) were created through the insertion of a stop codon into exon 5 of the CGRP/calcitonin gene, so calcitonin was still expressed normally (227). In contrast, the other strain of mice had exons 2–5 of the CGRP/calcitonin gene replaced with a PGK neoBPA cassette, which also knocked out the calcitonin gene. Although no evidence for calcitonin regulation of the cardiovascular system has been found to date, the loss of this protein may be relevant to the increased blood pressure observed in the knockout mice (108). A third strain of αCGRP knockout mouse, produced more recently, has added to the confusion surrounding the role of αCGRP in the maintenance of basal blood pressure (280). These mice expressed calcitonin normally, but still showed an increase in both mean arterial pressure and heart rate, compared with their wild-type counterparts. These cardiovascular changes were suggested to be a result of increased sympathetic nervous activity, which was measured as an increased level of urinary catecholamine metabolites (280). The reasons for the observed differences in these strains of mice remain to be elucidated.

The studies using the αCGRP knockout mice have been important in the absence of a well-characterized, potent, and selective antagonist for the CGRP receptor(s). They have revealed some inconsistencies compared with each other and to the antagonist studies, and these require further investigation. In comparison, it is established that $CGRP_{8-37}$ has little potency as a systemic vasoconstrictor, although effects observed on regional blood flow (110) suggest that CGRP may play a local modulatory/homeostatic role in the control of blood pressure. Furthermore, it has been suggested that the attenuated release of CGRP in spontaneously hypertensive rats may contribute to the observed hypertension (341).

B. AM: Role in Regulating Blood Pressure

AM has been shown to act as a vasodilator in a range of vascular beds and species (e.g., cat, rat, and sheep; Refs. 46, 51, 112) leading to decreased blood pressure and increased heart rate. In addition, AM has been shown to act via NO-dependent mechanisms to dilate the renal vasculature and to mediate diuretic and natriuretic responses in the kidney (239). The vascular relaxant effects of AM administration in the cerebral and systemic circulations are blocked by AM_{22-52} in the rat (76, 112). However, in a model of endotoxemia in the conscious rat, evidence was provided for a hypotensive role for CGRP, but not AM (112). In comparison, Mazzocchi et al. (245) have provided evidence for a role for AM, in a model involving intraperitoneal administration of endotoxin. The hypotensive responses were blocked by AM_{22-52}, but not $CGRP_{8-37}$. Others have suggested that AM may have a role in maintaining renal blood flow in septic shock (274). This is further discussed in section IX*E*.

Development of AM knockout mice has also been attempted, but surprisingly deletion of the AM gene in mice is lethal. The reason is thought to be due to insufficient development of blood vessels, umbilical arteries, and the formation of hydrops fetalis (40, 141). Studies of pregnant subjects have revealed that AM levels are raised in plasma, amniotic fluid, and cord blood from 8 weeks of gestation onwards (74). It is thought that AM may have an essential role in fetal development as well as the regulation of placental and fetal circulation (232, 254, 394).

Studies with transgenic mice overexpressing AM, and heterozygote AM knockout mice have provided evidence that AM may play a role in the regulation of basal blood pressure. The blood pressure in heterozygotes was elevated, with suppressed NO production (327), while the blood pressure in AM overexpressing transgenics was lower (326). In addition, these mice were more resistant to septic shock than wild-type mice (326), as discussed in section IX*E*. Furthermore, it has been clearly shown that in a model of cardiovascular injury, induced by salt loading, the heart weight-to-body weight ratio was significantly increased in heterozygotes. In addition, the perivascular coronary artery in heterozygotes contained inflammatory cells with a fibrotic appearance, and transforming growth factor-β (TGF-β) mRNA was upregulated. Thus it is suggested that AM possesses protective action against angiotensin II-induced coronary artery injury and cardiomegaly (95, 141).

The infusion of AM via the brachial artery in human volunteers is associated with dose-dependent vasodilatation and increased blood flow (63). Indeed, at low doses AM was considered to be more potent than CGRP. The response to administration in humans appears dose-dependent in that doses that produce a low plasma AM concentration (<12 pM) induce a long-lasting decrease in blood pressure without affecting plasma renin and catecholamine levels, or urine sodium content (212). However, infusions of higher amounts, leading to plasma levels of 50 pM or greater, are associated with tachycardia and increased prolactin levels.

VII. MICROVASCULAR MECHANISMS

A. CGRP: A Protective Factor?

The intravenous infusion of subvasodepressor doses into the conscious rat led to specific relaxant effects in a range of tissues, for example, a reduction in hindquarters vascular resistance (109). The concept of CGRP as a highly targeted vasodilator is enhanced by the observation of increased microvascular blood flow induced in the ipsilateral, but not contralateral, skin of the hindleg of the anesthetized rat after stimulation of CGRP-containing nerves, demonstrating that its activity is primarily at the site of release (92). It is also in keeping with the observation of selective facial flushing observed after intravenous CGRP administration in humans (113). Furthermore, in studies with genetically transformed mice, Champion et al. (43) have developed elegant techniques to study the vascular responses in the lung and hindquarters of the mouse. The wild-type and CGRP knockout mice used exhibited similar blood pressure, but vascular resistance was increased in knockout mice, suggesting that CGRP normally acts as a "braking system" for vascular resistance in the microvasculature.

The potent microvascular vasodilator activity of CGRP and its wide distribution in the periphery ensures that it is in a prime position to protect tissues from injury, in addition to regulating tissue blood flow under physiological conditions. Indeed, an involvement of CGRP-containing sensory nerves in the maintenance of tissue homeostasis has been proposed for some years. Evidence from a variety of tissues is given below. Of particular interest is the observation of both harmful and protective roles, depending on the circumstance of CGRP release. The administration of capsaicin to the rat at a neonatal stage leads to a selective destruction of capsaicin-sensitive sensory nerves, and thus a loss of their contents (e.g., CGRP). This is associated with an increased likelihood of the appearance of cutaneous lesions (236, 352). The beneficial role of sensory nerves is considered to be due to the vasodilator activity of CGRP. Peripheral vascular conditions associated with a deficit of CGRP-containing nerves, vascular dysfunction, and slow wound healing include diabetes (21) and Raynaud's disease (37), where a lack of reflex vasodilatation is observed. In comparison, excess release of CGRP is associated with blushing syndromes (388).

A rat skin flap model has allowed the demonstration that transcutaneous electrical stimulation is associated with increased blood flow and tissue perfusion in normal rats, but not in rats depleted of sensory nerves with capsaicin (200). Perhaps more importantly, there was a clear decrease in survival of the sensory nerve-depleted skin flaps, while addition of exogenous CGRP was found to increase skin blood flow and flap survival (199). In support of these studies, Knight et al. (201) demonstrated that infusion of a CGRP analog appears to rescue ischemic skin flaps. They demonstrated that CGRP infusions were associated with a restoration of tissue ATP levels and reduced thromboxane (a constrictor eicosanoid) levels. In comparison, CGRP did not affect either lipid peroxidation or neutrophil accumulation (201). However, a beneficial role of CGRP was clear.

In many species, the coronary arteries and left anterior descending artery receive innervation from a high density of CGRP-containing nerve fibers (104). CGRP is released from the heart in laboratory species in response to ischemia and low pH (105), with evidence that endothelium-derived prostacyclin (PGI_2) may also play a role in CGRP release (178). Infusion of CGRP was shown to attenuate and delay ischemia reperfusion arrythmias in the anesthetized rat (397), and these beneficial effects have been shown to be inhibited by $CGRP_{8-37}$ in vitro (222). After occlusion of the left anterior descending coronary artery and subsequent reperfusion, CGRP was found to improve the contractile function of the heart in an ischemia model in the dog (12). Indeed, there is also evidence for a protective role of endogenous CGRP in a myocardial infarction model in the pig (179); however, exogenously administered CGRP caused hypotension but no cardioprotection, as observed by a lack of reduction in the size of infarct size (180). The term *preconditioning* is used to describe the ability of a tissue to withstand severe ischemic attacks after exposure to previous brief ischemic episodes. Li et al. (221) suggest that CGRP is involved in ischemic preconditioning, possibly via protection of microvascular endothelial cells, and that the protective role of nitroglycerin may be related to stimulation of CGRP release (403; see also sect. IX*B*).

Evidence suggests that ischemic insult leads to the release of CGRP in the rat intestine (250, 348), which contributes in a proinflammatory manner to the reperfusion injury. It has been demonstrated that the CGRP contributes both to the mesenteric injury, especially the systemic hypotension and plasma leakage, in combination with endogenous kinins (233). Interestingly, $CGRP_{8-37}$

was able to attenuate leukocyte infiltration. In comparison, it has been shown in the rat stomach that either sensory nerve activation, or infusion of CGRP, acts with other mediators to mimic the protection against ischemia observed with preconditioning (287). Furthermore, it has been suggested that the delayed cardioprotection observed after intestinal ischemic preconditioning is mediated by endogenous CGRP in an NO-dependent manner as an NOS inhibitor abrogated the response (390).

B. AM: Heterogeneity in Response

The complexities of the mechanisms of AM-induced vascular dilation, as discussed in section v with respect to mechanisms and section vi with respect to cardiovascular regulation, are mirrored in the microvasculature. AM can act on either the CGRP or AM receptors and then mediate relaxation via one of several mechanisms, dependent on activation of adenylate cyclase or potassium channels, or through NO release.

The intravenous administration of AM is associated with skin flushing (249), highlighting the ability of AM to selectively mediate increased microvascular blood flow in the cutaneous microcirculation in a similar manner to CGRP. The microvascular activity of AM has been compared with that of CGRP in a variety of studies including in the rat mesentery (278), rat pulmonary circulation (71), and rat skin (132). In most cases, AM was found to be 10–80 times less potent than CGRP, and responses were found to be antagonized by $CGRP_{8-37}$, suggesting that microvascular vasodilatation is mediated by the CGRP receptor (e.g., in rat cutaneous microcirculation and rat isolated heart, Refs. 91, 132, 393). However, some tissue responses are inhibited by the weak antagonist AM_{22-52}, indicating that AM is acting via the AM receptor to mediate these effects. The rat cerebral microcirculation provides an example of tissue heterogeneity with respect to AM receptors. In arterioles AM responses are blocked by $CGRP_{8-37}$ (256). However, binding in cortical microvessels was blocked by AM_{22-52}, but not by $CGRP_{8-37}$ (202). In addition, in certain microvascular tissues, such as the human coronary arterioles, AM acts to relax vessels at least in part via a NO-dependent mechanism (349).

The production of AM in vascular cells in response to inflammatory cytokines, in tissues that include the microcirculation (60), is relevant to its contribution to inflammatory events such as septic shock, and this is discussed in section ix*E*. One of the most obvious mechanisms by which this occurs is through arteriolar vasodilatation, allowing a larger number of inflammatory cells to be exposed to the inflamed tissue, together with an increased intravascular pressure within the leaky postcapillary venules. The mechanisms by which AM can contribute to the vascular inflammation are discussed more fully in section viii*B*.

A study of congenital heart failure in the rat has suggested that the AM_1 receptor (i.e., CL and RAMP2) is increased in the heart, but not the kidney (356). Thus the failing heart may adapt and therefore be in a position to respond more selectively to AM than other tissues.

VIII. INFLAMMATION AND VASCULAR BIOLOGY

Traditionally the immune system was believed to function independently of outside regulation: in vitro studies demonstrated that antigens could stimulate the immune cells to proliferate, produce antibodies, attack foreign bodies, etc., all in isolation from other physiological signals. The discovery that mediators such as NO and the eicosanoids, released from nonimmune cells, are able to modulate immune responses has stimulated the search for other immunomodulators. Evidence is accumulating that CGRP and AM are also able to contribute to immune processes, and the realization that neuropeptides, including CGRP, contribute to the immune system has been recently discussed (213). The results of studies on neuropeptide/leukocyte interactions are sometimes contradictory, but they point to the existence of another level of control for immunocytes. Few experiments have been carried out with antagonists to date. However, in several cases the effects of CGRP on immune cells can be blocked by the selective antagonist $CGRP_{8-37}$, implying a direct effect via the CGRP1 receptor in immune regulation.

A. CGRP: Cellular Effects

The most basic way in which CGRP influences the activity of inflammatory cells is through its activity as a vasodilator. By increasing the blood flow to the area in which it is released, it also increases the number of circulating cells and amount of other chemotactic factors that are present. Evidence in the rabbit suggests that the main in vivo effects of CGRP in potentiating neutrophil accumulation are due to this vasodilator activity, allowing more neutrophils to enter the inflamed area and increasing the supply of chemotactic compounds (e.g., IL-1; Ref. 35). Studies in a mouse air pouch model found that the CGRP antagonist $CGRP_{8-37}$ was able to inhibit neutrophil accumulation in response to IL-1 treatment (1).

A range of in vitro studies complements the results from studies carried out in vivo. A study by Hartung and Toyka (135) suggests that exposure of endothelial cells to CGRP increases their expression of adhesion molecules for neutrophils. Evidence for a more direct role for CGRP has been harder to come by, and the available information lacks consistency. In 1992 Zimmerman et al. (404) demonstrated that CGRP at a concentration of 10 pM could enhance the adhesion of neutrophils to endothelial cells,

independently of the adhesion molecules CD11/CD18, L-selectin, E-selectin, or intracellular adhesion molecule. Other studies around the same time by other groups used particularly high doses of the peptide, which may not be relevant to the concentrations reached physiologically. Neutrophil adhesion to human umbilical vein endothelial cells was stimulated by exposure to CGRP (2 μM) independently of protein synthesis, but requiring increases in $[cAMP]_i$ (340). The increased adhesion was abolished by treatment with $CGRP_{8-37}$, indicating a selective effect of CGRP. Although one study found that CGRP (10 μM) activates human neutrophils (303), a later study from a different group showed that CGRP (1 fM to 1 μM) had no effect on neutrophil aggregation or chemotaxis (128). More recently, CGRP (10 μM) was found to inhibit both the production of superoxide and the increase in $[Ca^{2+}]_i$ induced by either SP or exogenous IP_3 in neutrophils, an effect blocked by application of $CGRP_{8-37}$ (343). CGRP also inhibited the expression of CD11b (a major integrin involved in chemotaxis) in human neutrophils after exposure to LPS or formyl-methionyl-leucyl-phenylalanine (fMLP) (253). Further studies are necessary to fully understand the effects of CGRP on neutrophils. Much of the information gained to date has come from studies using high neuropeptide concentrations so their physiological significance is doubtful. However, it is possible that these activities are important in disease states where CGRP levels are increased.

A greater number of studies have been carried out on the interactions between CGRP and lymphocytes, and it is generally considered that CGRP attenuates lymphocyte activity. The discovery of specific CGRP binding sites on lymphocytes suggests that CGRP can exert direct effects on them (246). CGRP was found to inhibit differentiation and immunoglobulin production by pre-B cells, whereas no activity on mature B cells was reported. Intraepithelial lymphocytes from the rat gut mucosal were also found to possess specific CGRP binding sites and express the mRNA for CL (129). Physiological concentrations of CGRP inhibited pre-B cell colony formation stimulated by IL-7 (98). The inhibitory effects of CGRP on pre-B cells are possibly mediated by activation of PKA and a consequent induction of c-*fos* and AP-1 activity (247). CGRP also inhibits proliferation by T lymphocytes in vitro (24, 361). This inhibition has been linked to inhibition of IL-2 production. IL-2 production and receptor expression are upregulated after antigen activation of T cells and are necessary for proliferation to occur (371).

Interestingly, in a recent study, Bracci-Laudiero et al. (26) found that activated B lymphocytes strongly express CGRP, compared with resting cells. This expression was inhibited by exposure to anti-nerve growth factor (NGF) antibodies, indicating that, as in sensory neurons, NGF regulates the synthesis of CGRP. This observation was supported by another study that identified constitutive production of both αCGRP and βCGRP by human lymphocytes (372). Expression of βCGRP was increased by exposure to lymphocyte mitogen (such as phytohemagglutinin). The physiological significance of lymphocyte-derived CGRP is unclear.

The general inhibitory effects of CGRP on lymphocyte populations are surprising when considered alongside the many proinflammatory effects of CGRP in vivo, even in conditions featuring lymphocyte activity such as allergic contact dermatitis. CGRP-containing nerves were found to increase in a mouse model of contact dermatitis, and the release of CGRP stimulated increased leukocyte recruitment (123). It is possible that CGRP does exert an overall proinflammatory effect, even though its actions at the level of individual cells seem to oppose an immune response, by strictly controlling the rates of mitosis and differentiation exhibited by T cells during an inflammatory reaction. The initial T cell division would be triggered by exposure to antigen, and then enhanced by other inflammatory mediators. By preventing further progression of the T cells through the cell cycle, CGRP could cause them to terminally differentiate, forming effector T cells. In this way, an "anti-inflammatory"/inhibitory effect of CGRP would actually enhance the immune response, by stimulating the maturation of a population of resting T cells into effector T cells. An ability of CGRP to selectively suppress T cell proliferation and the synthesis of T_H1 cytokines has recently been correlated with prevention of B cell destruction and protection against the induction of insulin-dependent diabetes in the mouse (339). This may be an extremely important observation if confirmed in vivo.

A CGRP receptor has also been identified on macrophages derived from bone marrow. The mRNAs coding for CL and RAMP, the minimum component of a functional CGRP receptor, were found in these cells (97). Osteoclasts are thought to be derived from the monocyte/macrophage line, so the ability of CGRP to inhibit bone resorption/stimulate bone formation is probably related to a direct action on osteoclasts (149, 285). Exposure of macrophages to CGRP suppressed their production of IL-1 (355). It also inhibited antigen presentation by murine macrophages and the oxidative burst response in human blood monocytes (276). Torii et al. (355) further investigated the decrease in antigen presentation induced by CGRP in a study on human macrophages. They found that the decrease was associated with reductions in the production of IL-12 and the expression of B7.2 at the cell membrane. This protein is one of the two isoforms of B7, found on antigen-presenting cells, which binds to CD28 on T cells. In this way, a decrease in expression of B7.2 leads to fewer macrophage/T cell interactions and thus decreases functional antigen presentation (355). The attenuation of IL-12 production by murine macrophages exposed to LPS is mediated by an increase in cAMP and

activation of PKA, associated with a decrease in IL-12 mRNA (223). CGRP has also been shown to have inhibitory effects on the antigen-presenting activity of Langerhans cells in the skin (41, 155).

Although the majority of effects of CGRP on lymphocytes and neutrophils are inhibitory, its actions on monocytes/macrophages are a mixture of both stimulatory and inhibitory effects. CGRP was found to upregulate IL-10 production by macrophages (355), providing a possible explanation for the inhibition of IL-2 production by CGRP. IL-2, primarily produced by T_H1 cells, is one of the key stimulatory cytokines on proliferation of antigen-stimulated T cells. IL-10, produced mainly by macrophages and T_H2 cells, inhibits the activity of T_H1 cells. Thus, by stimulating IL-10 release by macrophages, CGRP favors T_H2 (humoral) over T_H1 (cytotoxic) responses, suggesting that it may direct the immune system toward antibody-mediated responses. CGRP has also been shown to potentiate the LPS-induced release of IL-6 from murine macrophages, but has no effect on IL-6 production by unstimulated macrophages (345). In a further set of experiments by the same group, the potentiation was shown to be due to CGRP enhancing the production of NO and prostacyclin, which are stimulated by exposure to LPS. It is the elevated levels of NO and PGI_2 that then potentiate the release of IL-6 (347). Exposure to CGRP was also found to stimulate expression of IL-6 by bone marrow macrophages (97). The ability of CGRP to enhance NO production by LPS-stimulated macrophages was also observed in another study, where it was linked to an increase in iNOS expression (224).

In addition to the modulatory effects of CGRP in the cellular phase of inflammation, a number of studies in knockout mice suggest that it is also involved in the inflammatory hyperalgesia associated with vascular inflammation. CGRP knockout mice, in which both calcitonin and αCGRP have been deleted, are less sensitive to thermal hyperalgesia detected in the hindpaw in response to kaolin- or carrageenan-induced knee joint inflammation (399). In support of these findings, αCGRP knockout mice where only αCGRP expression is deleted have been shown to produce a significantly smaller response to a range of inflammatory/hyperalgesic stimuli when injected and studied in the hindpaw. These include capsaicin, Formalin, and carrageenan (312). No differences between wild-type and knockout mice were observed in tail flick (a spinal pain pathway) or hot plate (involving brain pain pathways) tests (311). This implies that CGRP is involved in processing of pain during ongoing inflammation, but not in the absence of inflammatory stimuli.

B. AM: Cellular Effects

The role of AM in the inflammatory and immune response is less clear than that of CGRP, as little research has been carried out into this aspect of its activity. AM, as discussed above, is well placed to contribute to the inflammatory process as its production is upregulated in a range of vascular and immune cells in response to cytokines. These cells include polymorphonuclear leukocytes, lymphocytes, monocytes, monocyte-derived macrophages, keratinocytes, cardiac myocytes, and fibroblasts (207, 208, 251), in addition to vascular smooth muscle and endothelial cells.

AM, like CGRP, is a potent microvascular vasodilator and so will enhance inflammatory and immune responses through an increase in blood flow to an inflamed area, thus increasing the supply of inflammatory mediators and leukocytes. The ability of both CGRP and AM to potentiate the plasma extravasation induced by other inflammatory mediators (e.g., substance P, bradykinin), without themselves producing edema, was demonstrated by Chu et al. (59). However, it has been suggested, mainly from studies on endothelial cells in culture, that AM may act, depending on circumstance, to inhibit endothelial hyperpermeability (146). This inhibitory process is linked to accumulation of cAMP in the endothelial cells (146). Chu et al. (59) also demonstrated that, in rat skin, AM potentiates neutrophil accumulation to IL-1β. This may be a direct effect on the neutrophils, rather than an indirect effect through vasodilatation, as the potentiating dose was lower than that which enhanced edema to substance P or bradykinin (59). However, AM has been shown to suppress fMLP-induced upregulation of the major human neutrophil adhesion molecule CD11b. The effect was secondary to an increase in cAMP that was blocked by $CGRP_{8-37}$, suggesting that this effect was mediated via the CGRP receptor (309).

AM has been shown to have definite direct effects, both stimulatory and inhibitory, on other inflammatory cells. Macrophages secrete neutrophil chemoattractants, particularly in the lung. AM inhibits this release from alveolar macrophages treated with LPS (182). It also inhibits IL-1β-induced TNF-α secretion and gene transcription from Swiss 3T3 fibroblasts (166), and LPS-induced TNF-α expression and release from a murine macrophage-like cell line (387). Both these activities are anti-inflammatory, and probably related to the ability of AM to increase cAMP, but a recent study suggests that AM may also have direct pro-inflammatory effects. Yoshida et al. (392) showed that AM (10^{-8}–10^{-5} M) dose-dependently increased histamine release from a preparation of peritoneal mast cells, an effect which was blocked by treatment with the weak, but selective antagonist AM_{22-52}. However, this is a particularly high concentration of AM, so the physiological relevance of this phenomenon is unproven.

In addition to its ability to modulate the immune response via interactions at a cellular level, AM may play an even more direct role. It is expressed in the epithelial surfaces that provide a barrier to pathogen entry into the

body (e.g., skin, lung, gut, oral cavity). There is some evidence to suggest that AM can kill, or inhibit, the growth of Gram-positive and Gram-negative bacteria at these surfaces (6). Although this requires a concentration higher than physiological circulating levels, these concentrations may be achieved in certain circumstances (e.g., sepsis) and so allow a response to pathogen challenge.

IX. INVOLVEMENT IN CARDIOVASCULAR DISEASE

The previous sections have highlighted the similarities in the cardiovascular activities of CGRP and AM. Their involvement in different clinical conditions arises mainly from their different sites of release. CGRP and AM have been linked to a range of pathophysiological conditions, and the role of amylin in diabetes has been reviewed elsewhere (154, 206). This section provides an account of the role of CGRP and AM in cardiovascular diseases with evidence coming from a range of studies, and where agents that modulate the activity of the peptide may have potential as novel therapeutic approaches.

A. CGRP and Cerebral Conditions

The most compelling evidence of a role for CGRP in a pain syndrome comes from sufferers of migraine and cluster headache. Studies have shown that CGRP levels are raised during the painful phases of both conditions (120, 122) and are restored to basal levels by successful migraine treatment with triptan 5-HT_1 agonists, providing evidence that the trigeminal sensory nervous system is activated (260). The intracranial extracerebral blood vessels (e.g., middle meningeal artery and its dural arterioles) which supply the dura mater are thought to dilate and as a consequence stimulate perivascular sensory nociceptive nerve fibers, producing a sensation of pain (118). It is now known that exogenous CGRP induces a delayed migraine-like headache in migraineurs (215), but there is little evidence for a role of CGRP in tension-type headache (11). The release of CGRP, and the role of the blood-brain barrier, in migraine are still poorly understood. CGRP can be released from either the trigeminal ganglia or perivascular nerves. It has recently been suggested that triptans act by causing a prolonged elevation of intracellular calcium in trigeminal neurons, which blocks the MAPK activation of CGRP synthesis and release (82). 5-HT_1 agonist treatment of migraine is associated with a rebound effect; the pain is removed, but then returns several hours after the initial attack (321). This has spurred on the search for a new class of antimigraine drugs. The nonpeptide CGRP antagonist BIBN4096BS has been tested in phase II clinical trials as a potential novel treatment (78) and found to be effective against the symptoms of migraine, without significant acute side effects (84).

Subarachnoid hemorrhage is associated with cerebral vasoconstriction that occurs several days after the hemorrhage and is often fatal. The vasospasm occurs in 30–40% of patients and is the major cause of death from this condition. The vasoconstriction is associated with a decrease in CGRP levels in nerves (85) and an increase in CGRP levels in draining blood (175), suggesting that CGRP is released from nerves to oppose the vasoconstriction. This evidence has led to the concept that addition of CGRP may be beneficial in a condition that has proven hard to treat. A preliminary clinical trial with CGRP provided evidence for an ability of intravenously administered CGRP to reverse the vasoconstriction (174). However, this was not reflected in a multicenter clinical trial (18). It has been shown that gene transfer of recombinant adenoviral preproCGRP can prevent fatal cerebral vasoconstriction after subarachnoid hemorrhage in a rabbit model (357). It is therefore possible that gene therapy may be appropriate in this condition and of greater benefit than current therapies or intravenous infusion of CGRP, although mechanisms for the administration of such treatments will have to be developed.

B. CGRP and AM: Heart Conditions

The heart is innervated by CGRP-containing fibers, and in humans there is immunohistochemical evidence for CGRP in nerves that innervate coronary arteries, with distal arteries (<0.8 mm ID) receiving a denser innervation (127, 338). The local release of CGRP from cardiac and coronary tissues is thought to counteract the effects of ischemic episodes leading to a cardiac protective effect (218, 238). Animal models support this concept, as discussed in section VIIA. After acute myocardial infarction in humans, there is an increase in immunoreactive CGRP in plasma, as well as in nerves (suggesting ischemia-induced upregulation) (296, 306). Evidence indicates that CGRP can have a protective influence in dilating coronary arteries at locations of atheromatous stenosis, and delaying the onset of myocardial ischemia in patients with chronic angina undergoing treadmill exercise (364).

The infusion of CGRP is beneficial in increasing cardiac output and lowering blood pressure in patients with congestive heart failure (114, 322); however, more recent studies have concentrated on the role of AM. Production of AM by vascular cells and myocytes is central to evidence described below which suggests that AM may have a role in the pathophysiology of a range of cardiovascular conditions. Furthermore, it has been suggested that centrally produced AM may also be relevant to cardiovascular regulation (244, 314).

Congestive heart failure is associated with increased plasma levels of AM that are related to disease severity, as

measured in plasma by several groups (171, 187, 272, 344), and AM levels decrease after successful treatment (272). It is suggested that the AM is produced as part of a physiological defense mechanism to counteract vasoconstriction. Interestingly, in the failing heart, AM expression was found to be increased in the ventricular myocytes (171). In addition, it is suggested that plasma AM levels are correlated with diastolic dysfunction in these patients (395). The vasodilatation observed in response to local infusion of AM into the human forearm via the brachial artery was reduced in patients with congestive heart failure (269). This suggests a lack of effect of AM in these patients, and there have now been studies where AM was administered to patients with impaired left ventricular systolic function after acute myocardial infarction. AM administration led to a fall in arterial pressure, and thus reduced cardiac work, with a minimal effect on urine sodium excretion (50). A second study supports the finding that AM reduces the cardiac work load, although in this case along with increased urinary volume and sodium excretion (267). Thus it is possible that the administration of AM may be of use in the treatment of congestive heart failure. Alternatively, it is possible that endogenous levels of AM could be increased by use of neutral endopeptidase inhibitors, as demonstrated in the human forearm (381). However, a potentiation of AM responses by neutral endopeptidase inhibitors was not observed when resistance arteries from patients with chronic heart failure were studied in vitro (289).

C. AM and Hypertension

Plasma levels of AM are correlated with blood pressure increase in hypertensive patients, and thus with disease severity (165, 196, 203). There is a link between hypertension and impaired kidney function, and plasma levels of AM are also positively coupled with impaired renal function and renal failure (165, 203). In addition, there is a relationship between AM levels and cardiac and arterial hypertrophy (257, 337). This suggests a direct relationship between AM release and increased blood pressure. It is possible that AM is released as a protective mechanism to counteract increasing blood vessel tone, as described above. However, it has also been noted that plasma AM levels remain high even in patients with effective antihypertensive therapy (203), and interestingly, the levels of AM do not appear to be altered by changes in salt levels (164). Although plasma AM levels increase with pregnancy (217, 241), there is no clear role for AM as a defense against preeclampsia (169).

The hypothesis that AM may be of benefit as an antihypertensive agent has led to gene therapy studies, with AM delivered by an adenoviral vector. This regime led to a substantially reduced blood pressure for 9 days in a spontaneous hypertensive, Dahl salt-sensitive, deoxycorticosterone acetate-salt (DOCA) model of hypertension in the rat (49, 398). These results cannot be directly translated to humans as the viral vector induces inflammation via activation of the immune system leading to release of AM from nontransfected sites (290). However, gene therapy with CGRP and CGRP-like peptides as a treatment for hypertensive disorders remains an exciting possibility. This hypothesis is supported by studies that show that infusion of AM lowers blood pressure in models of hypertension (194, 195) and that AM inhibits hypertrophy of cultured cardiomyocytes (359). The effect of AM in patients with essential hypertension has also been investigated. To mimic the plasma concentrations observed in advanced cardiovascular disease, AM was infused intravenously (358). Short-term AM infusion was found to induce vascular relaxation and decreased systolic and diastolic blood pressure. An increase in heart rate was observed, possibly related to sympathetic activation as plasma catecholamine levels rose. In addition, angiotensin II levels were raised. Thus the study provided evidence for a link with the renin-angiotensin system and the sympathetic constrictor system. Skin flushing was observed, and this may have accounted for some of the hypotension observed.

A link has been recently suggested between a microsatellite CA repeat polymorphism in the DNA adjacent to the AM gene and genetic predisposition to essential hypertension. Four alleles of this polymorphism were identified, with 11, 13, 14, and 19 repeats. Patients with essential hypertension were twice as likely to possess the 19 CA repeat as the normotensive subjects examined (162). The relevance of this is not yet known, and this is one of several polymorphisms that has been linked with hypertension. It may be that multiple polymorphisms increase the likelihood of predisposition to hypertension (286).

D. CGRP and AM: Pulmonary Hypertension

CGRP is believed to play an important role in maintaining low pulmonary vascular resistance under physiological conditions. Pulmonary hypertension is due to a local constriction of arterioles in the lung. It is thought that local vasodilator mechanisms become deficient due to either insufficient production of endogenous vasodilators or excessive production of vasoconstrictors. Recent results reveal upregulation of RDC-1, RAMP1, and RAMP3 mRNAs in rat hypoxic lung with no change in levels of CL and RAMP2 mRNAs (297). These findings are in keeping with a vasodilator role for CGRP and AM in the pulmonary circulation. Indeed, it has been shown that CGRP acts in a potent manner to dilate precontracted pulmonary arteries in vitro (243). The rat provides an excellent model of hypoxic pulmonary hypertension. Application of

exogenous CGRP prevents the development of, and reverses existing, hypoxic pulmonary hypertension (353, 354). In addition, the increase in pulmonary hypertension correlates well with declining blood CGRP levels in the rat (191), and depletion of CGRP from sensory nerves by capsaicin pretreatment exacerbates hypoxia-induced pulmonary hypertension in rats (354).

A mouse model of chronic hypoxia has recently been studied where an adenoviral vector carrying prepro-CGRP was employed to deliver CGRP-encoding DNA to the lungs via intratracheal tube. The increases in pulmonary vascular resistance, right ventricular mass, and pulmonary vascular remodelling after hypoxia were then compared with those in mice without CGRP transfection. The lung CGRP and cAMP levels were increased, while all the measured parameters were decreased without any decrease in systemic blood pressure. Thus CGRP gene transfer to the lung attenuates pulmonary hypertension symptoms in chronically hypoxic mice, indicating that CGRP gene transfer alone may have a beneficial role (44). An adenoviral vector encoding prepro-CGRP was also transfected into the lungs of CGRP knockout mice (43), with a similar beneficial outcome. These results suggest that gene transfer of CGRP alone or concomitantly with a cAMP phosphodiesterase inhibitor may represent a new strategy in the treatment of disorders such as pulmonary hypertension. However, in a study of five patients with primary pulmonary hypertension, CGRP failed to alter pulmonary arterial pressure, total pulmonary vascular resistance, or cardiac output after infusion into the right atrium, possibly because of impaired endothelium-dependent vasodilatation (363).

AM is also a candidate for treatment in this disease due to its powerful vasodilator activities in the peripheral microcirculation (369). Like CGRP, AM acts to reduce pulmonary artery pressure in the rat lung during hypoxia-induced pulmonary hypertension in a $CGRP_{8-37}$-sensitive manner, suggesting that AM acts via the CGRP receptor in this model (401). Interestingly, in the cat, it is suggested that AM has a greater effect than CGRP in the pulmonary microcirculation, an indication that AM acts on receptors distinct from those of CGRP in this species (328). Plasma levels of AM are increased in patients with pulmonary hypertension, in a direct relationship with disease severity (177). Nagaya et al. (266) have investigated the effect of AM infused intravenously. Their results suggest that there was little effect on systemic blood pressure, but pulmonary and systemic vascular resistance fell, with a beneficial effect on arterial oxygen levels. There were associated increases in brain-derived natriuretic peptide and aldosterone concentrations. It has also been suggested that AM may play a role in inhibiting pulmonary vascular remodeling, as it is released by and inhibits proliferation of human pulmonary artery smooth muscle cells (362).

E. CGRP and AM: Sepsis

Polymicrobial sepsis is the systemic response to an infectious process. It consists of an early hemodynamic phase, with increased cardiac output and tissue perfusion, followed by a secondary hypodynamic phase with decreased tissue perfusion and hypotension. It often leads to multiple organ failure and death. It is considered that a range of inflammatory mediators are involved in these responses. Both CGRP and AM have been implicated in sepsis and are thought to be involved in the pathology. CGRP levels have been shown to be raised in septic shock in rats (346) and humans (172). AM is present from an earlier time point than CGRP and may play a pivotal role in the transition from the first to the second phase of sepsis (204, 373). Certainly, levels of AM are raised during sepsis in humans (147, 274). The upregulated levels of AM have been shown to be directly related to circulating endotoxin in the rat (391), and the small intestine has been demonstrated to be a major site of AM synthesis and release during sepsis (402). Indeed, transgenic mice overexpressing AM were found, in an LPS model of septic shock, to show resistance to hypotension and liver damage, with improved survival compared with wild-type mice (326). It has been suggested that the AM receptor acts as a clearance receptor for AM in sepsis (283, 284). During sepsis, decreased receptor levels or saturation of the receptor in the lungs leads to raised levels of circulating AM (283, 284). This is supported by a study carried out in the rat isolated lung, which demonstrates that the weak AM receptor antagonist AM_{22-52} but not the CGRP antagonist $CGRP_{8-37}$ enhanced levels of AM in the perfusate (80).

A similar systemic condition is often seen in the absence of infection and called SIRS. This condition is seen after injury that includes burns and surgical procedures. In a similar manner to the pathologies described above, AM plasma levels were found to correlate with the severity of symptoms (360). Interestingly TNF-α levels were found to correlate with AM levels, suggesting a specific link between these mediators in this condition. It is possible that TNF-α is responsible for stimulating the upregulation of AM in SIRS.

F. CGRP and Raynaud's Disease

A role for CGRP in the pathology of Raynaud's disease has long been suspected. This syndrome is characterized by severe episodic peripheral vasospasm, particularly affecting the hands and feet. The loss of blood flow leads to chronic pain and, in some cases, ulceration, infection, and gangrene that may necessitate amputation. In normal individuals the cutaneous microvasculature is densely innervated by CGRP-con-

taining nerves (116), so there are high levels of CGRP in the digits. Immunohistochemical analysis has revealed two distinct populations of these nerves. The most common (75%) contain CGRP and somatostatin, whereas the remainder contain CGRP and substance P (116). However, sufferers of Raynaud's disease are found to have a deficiency in CGRP levels within the perivascular nerves, coupled to an increased sensitivity of the dermal microvasculature in the hands to systemic CGRP (38, 319, 351). Intravenous administration of CGRP leads to peripheral vasodilatation and promotes the healing of ulcers in Raynaud's sufferers (37, 320). It is suggested that CGRP may be at least as effective as other agents used for treatment (nitroglycerin and PGI_2), but more appropriate routes via which CGRP may be administered are required.

G. CGRP and Blushing Syndromes

The opposite condition to that seen in Raynaud's disease, an increase in CGRP release, is believed to be involved in the onset of blushing syndromes. Estrogen levels are decreased (leading to a relative increase in androgens) in peri- and postmenopausal women, who commonly suffer from uncomfortable and embarrassing hot flushes and episodic sweating. These can be alleviated by hormone replacement therapy (225). The flushing is believed to be due to an increase in release of CGRP, which is found to be elevated in serum and urine during attacks, although the tachykinins which colocalize with CGRP are not found to increase (53, 389). A similar condition is seen in males with a loss in circulating androgens, after surgical removal of the prostate gland in prostate cancer. In this case, it seems to be the relative increase in estrogens that leads to hot flushes, where again an increase in plasma CGRP is observed (329). These studies suggest that sex hormone levels are linked to the production/release of CGRP, although the precise mechanisms remain to be determined.

X. CONCLUSION AND FUTURE PERSPECTIVES

This review has summarized, and attempted to correlate, the cardiovascular activities of CGRP and the related peptides AM. Figure 4 provides a summary of the comparative expression, release, and receptors for CGRP and AM, the two peptides of greatest cardiovascular relevance. There have been previous reviews on the cardiovascular activities of the separate peptides (e.g., Refs. 20, 28, 95, 170) or on the cellular aspects that relate to this fascinating peptide family (365, 383). However, it is only when the cardiovascular activities of these peptides are discussed simultaneously that the similarities in activity become clear and that themes of potential importance emerge. The most important facet of their activity is their potency as peripheral vasodilators. This suggests that, at least in terms of CGRP, they probably play an important role in the regulation of tissue perfusion, possibly acting as a "braking system" as described in section VI. This microvascular potency also leads to the characteristic flushing that is observed upon the intravenous administration of either CGRP or AM. The latter effect emphasizes the difficulty that would be faced in using the systemic administration of CGRP or AM as a routine therapeutic approach for the treatment of cardiovascular disease. The finding does demonstrate, however, the potential ability of AM to disrupt homeostasis if sufficiently upregulated in systemic disease such as septic shock.

One of the major advances in recent years has been the synthesis of nonpeptide molecules that are capable of antagonizing effects mediated via the CGRP receptor. Indeed, as discussed in sections IV and IX, there is currently a compound in clinical trials for migraine therapy. There is compelling evidence for a role for CGRP in the pathology of migraine, and the results of these trials are eagerly awaited. However, in keeping with all other new drugs, a lack of side effects is essential. Thus an essential component in the drug development is a requirement to prove that the CGRP antagonist is not instrumental in producing/worsening other illnesses, such as Raynaud's syndrome, pulmonary hypertension, ischemia/reperfusion injury, or congestive heart failure as a result of decreased CGRP activity. Interestingly, other possible outcomes such as inhibition of facial flushing may be considered an advantage, rather than a disadvantage of the drug.

Knowledge of the roles of CGRP and AM in physiology and pathology has increased substantially in recent years. However, one area that remains unclear is the extent to which the CGRP receptor is utilized by AM. There is evidence, as extensively discussed in the previous sections, that AM can either act on the CGRP or AM receptor, depending on tissue and species (e.g., Refs. 132, 202, 278, 349, 393). A better understanding of the receptor-coupling mechanisms of AM will, without doubt, lead to further discussion of the potential therapeutic utility of ligands that modulate the activity of this receptor. Clearly, the evidence of the essential role of AM in fetal development (40, 141) may limit the therapeutic potential of future AM antagonists.

Finally, there is also potential for CGRP and AM agonists to offset the adverse effects of constriction and ischemia, as observed in pulmonary hypertension (266, 354) and myocardial infarction (218, 306, 364). The requirement for local administration of these peptides means that targeted gene delivery may be a particularly relevant method of treatment and the experiments de-

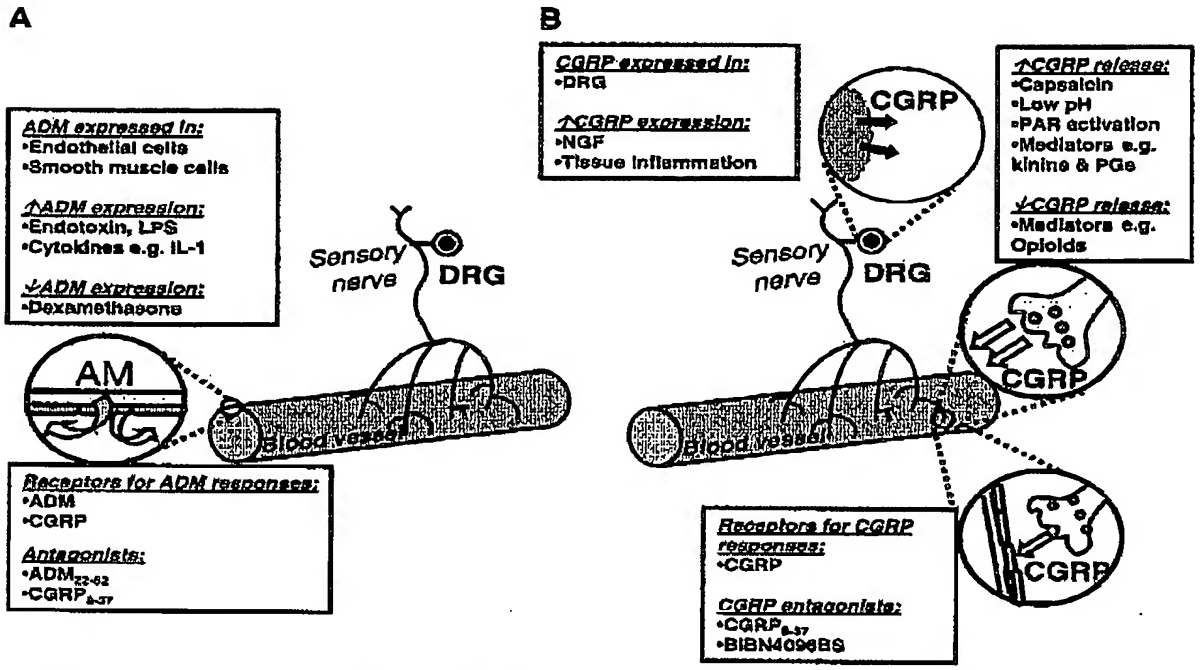

FIG. 4. A diagram of two sensory nerve-innervated blood vessels designed to show comparative sites of CGRP and AM expression, release, and activity. Some areas have been enlarged for added clarity, as indicated by the circles and are linked to text boxes that provide explanation. *A*: the sensory nerve-independent release and activity of AM. The AM gene is expressed and upregulated in a range of tissues including vascular smooth muscle and endothelial cells. The expression is increased by endotoxin [e.g., lipopolysaccharide (LPS)], cytokines [e.g., interleukin (IL)-1] and stress (e.g., shear stress). Anti-inflammatory steroids (e.g., dexamethasone) decrease AM expression. AM can signal via both AM and CGRP receptors. Both AM receptor antagonists (e.g., AM_{22-52}) and CGRP receptor antagonists (e.g., $CGRP_{8-37}$) can antagonize AM responses. *B*: the sensory nerve-dependent mechanisms of CGRP release and activity. The CGRP gene is expressed in the dorsal root ganglion (DRG) and is upregulated by factors that include nerve growth factor (NGF) and tissue inflammation. CGRP is released from nerves in response to a number of stimuli, such as capsaicin and low pH (via TRPV1 receptors), proteinase-activated receptor (PAR activation), and mediators (e.g., kinins and prostaglandins). The release can be inhibited by factors that include opioids. CGRP acts via the CGRP receptor. Responses to CGRP are inhibited by CGRP receptor antagonists.

scribed so far in models of pulmonary hypertension and subarachnoid hemorrhage can only act to stimulate further interest in this area.

In conclusion, this review acts to integrate evidence from recent developments in molecular and cardiovascular research to present evidence for a pivotal role of CGRP, AM, and amylin in the physiology and pathophysiology of cardiovascular regulation. Research in this field is at an exciting stage where selective ligands are in the process of clinical trials for use as selective therapies, in migraine. It will be interesting to follow the success of such compounds especially when considering the pleiotropic nature of the CGRP family of peptides.

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